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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSING AND TREATING DISORDERS INVOLVING ANGIOGENESIS

(57) Abstract: The present invention relates to polynucleotides associated with angiogenesis-related disorders. The present invention also relates to feline angiostatin genes, novel genes associated with angiogenesis-related disorders, such as cancer. The invention encompasses angiostatin nucleic acids, recombinant DNA molecules, cloned genes or degenerate variants thereof, angiostatin gene products and antibodies directed against such gene products, cloning vectors containing mammalian angiostatin gene molecules, and hosts that have been genetically engineered to express such molecules. The invention further relates to methods for the identification of compounds that modulate the expression of angiostatin genes and gene products and to using such compounds as therapeutic agents in the treatment of angiogenesis-related disorders, e.g., cancer. The invention also relates to methods for the diagnostic evaluation, genetic testing and prognosis of angiogenesis-related disorders, e.g., cancer and to methods and compositions for the treatment of these disorders.

tumor, but also for the dissemination and recolonization of metastatic tumor cells. The precise mechanisms that control the angiogenic switch is not well understood, but it is believed that neovascularization of tumor mass results from the net balance of a multitude of angiogenesis stimulators and inhibitors (Folkman, 1995, *Nat Med* 1(1):27-31).

5 One of the most potent angiogenesis inhibitors is angiostatin identified by O'Reilly and Folkman (O'Reilly et al., 1997, *Cell* 88(2):277-85; O'Reilly et al., 1994, *Cell* 79(2):3 15-28). Its discovery was based on the phenomenon that certain primary tumors can inhibit the growth of distant metastases. O'Reilly and Folkman hypothesized that a primary tumor initiates angiogenesis by generating angiogenic stimulators in excess of inhibitors. However,
10 angiogenic inhibitors, by virtue of their longer half life in the circulation, reach the site of a secondary tumor in excess of the stimulators. The net result is the growth of primary tumor and inhibition of secondary tumor. Angiostatin is an example of a growing list of such angiogenesis inhibitors produced by primary tumors. Angiostatin is a proteolytic fragment of a larger proteins: angiostatin is a 38 kDa fragment from plasminogen (amino acid Val₉₈-Gly₄₅₉ in murine plasminogen). Angiostatin has been shown to specifically inhibit endothelial cell
15 proliferation in vitro and block angiogenesis in vivo. More importantly, administration of angiostatin to tumor-bearing mice leads to significant tumor regression, and no toxicity or drug resistance has been observed even after multiple treatment cycles (Boehm et al., 1997, *Nature* 390(6658):404-407). The fact that angiostatin targets genetically stable endothelial
20 cells and inhibits a variety of solid tumors makes it a very attractive candidate for anticancer therapy (Fidler and Ellis, 1994, *Cell* 79(2):185-8; Gastl et al., 1997, *Oncology* 54(3): 177-84; van Hinsbergh et al., 1999, *Ann Oncol* 10 Suppl 4:60-3). In addition, angiogenesis inhibitors have been shown to be more effective when combined with radiation and chemotherapeutic agents (Klement, 2000, *J. Clin Invest*, 105(8) R15-24. Browder, 2000, *Cancer Res.* 6-(7)
25 1878-86, Arap et al., 1998, *Science* 279(5349):377-80; Mauceri et al., 1998, *Nature* 394(6690):287-91).

Cancer is not only devastating to humans, but is also the most common cause of natural death in cats. There are 1277 tumor bearing cats per 100,000. (William A. Prester and Frank W. McKay - Malignant tumor types in companion animals: the Occurrence of tumors in
30 domestic animals. NCI monograph # 54 1980. NIH Publication No. 80-2046). Treatments such as radiation therapy and chemotherapy, have only very limited success. Thus, more effective treatments for angiogenic diseases, such as, for example, feline cancers, are necessary.

SUMMARY OF THE INVENTION

35 The present invention encompasses novel nucleotide sequences that are associated with angiogenesis related disorders, e.g., cancer. The invention more specifically relates to nucleotide sequences that encode angiostatin. In addition, angiostatin nucleic acids, recombinant DNA molecules, cloned genes or degenerate variants thereof are provided herein. The invention also provides vectors, including expression vectors, containing

METHODS AND COMPOSITIONS FOR DIAGNOSING AND TREATING
DISORDERS INVOLVING ANGIOGENESIS

FIELD OF THE INVENTION

The present invention relates to polynucleotide sequences which are shown herein to be associated with the regulation of angiogenesis. More specifically, the present invention relates to novel polynucleotide sequences which encode the angiogenesis inhibitor angiostatin; more particularly, the feline angiostatin. The invention encompasses angiostatin nucleic acids, recombinant DNA molecules, cloned genes and degenerate variants thereof, vectors containing such angiostatin nucleic acids, and hosts that have been genetically engineered to express and/or contain such molecules. The invention further relates to angiostatin gene products and antibodies directed against such gene products. The invention further relates to methods for the identification of compounds that modulate the expression, synthesis and activity of such angiostatin nucleic acids, and to methods of using compounds such as those identified herein as therapeutic agents in the treatment of angiogenesis-related disorders, including, but not limited to, cancer. The invention also relates to methods for the diagnostic evaluation, genetic testing and prognosis of an angiogenesis-related disorder, including, but not limited to, cancer.

BACKGROUND OF THE INVENTION

Angiogenesis, defined as the growth or sprouting of new blood vessels from existing vessels, is a complex process that primarily occurs during embryonic development. Under normal physiological conditions in adults, angiogenesis takes place only in very restricted situations such as hair growth and wounding healing (Auerbach, W. and Auerbach, R., 1994, *Pharmacol Ther* 63(3):265-311; Ribatti et al., 1991, *Haematologica* 76(4):311-20; Risau, 1997, *Nature* 386(6626):671-4). Unregulated angiogenesis has gradually been recognized to be responsible for a wide range of disorders, including, but not limited to, cancer, cardiovascular disease, rheumatoid arthritis, psoriasis and diabetic retinopathy (Folkman, 1995, *Nat Med* 1(1):27-31; Isner, 1999, *Circulation* 99(13):1653-5; Koch, 1998, *Arthritis Rheum* 41(6):951-62; Walsh, 1999, *Rheumatology (Oxford)* 38(2):103-12; Ware and Simons, 1997, *Nat Med* 3(2):158-64). Of particular interest is the observation that angiogenesis is required by solid tumors for their growth and metastases (Folkman, 1986, *Cancer Res*, 46(2):467-73. Folkman 1990, *J Natl. Cancer Inst.*, 82(1):4-6, Folkman, 1992, *Semin Cancer Biol* 3(2):65-71; Zetter, 1998, *Annu Rev Med* 49:407-24). A tumor usually begins as a single aberrant cell which can proliferate only to a size of a few cubic millimeters due to the distance from available capillary beds, and it can stay 'dormant' without further growth and dissemination for a long period of time. Some tumor cells then switch to the angiogenic phenotype to activate endothelial cells, which proliferate and mature into new capillary blood vessels. These newly formed blood vessels not only allow for continued growth of the primary

angiostatin nucleic acid molecules, and hosts that have been genetically engineered to express and/or contain such angiostatin gene products.

The invention further relates to novel angiostatin gene products and to antibodies directed against such gene products, or variants or fragments thereof.

5 The invention further relates to methods for modulation of angiostatin-mediated processes and for the treatment of disorders involving angiogenesis, such as cancer, including the amelioration or prevention of at least one symptom of the disorders, wherein such methods comprise administering a compound which modulates the expression of an angiostatin gene and/or the synthesis or activity of an angiostatin gene product. In one
10 embodiment, the invention relates to methods for the use of a novel angiostatin gene product or fragment, analog, or mimetic thereof, or an antibody or antibody fragment directed against an angiostatin gene product, to treat or ameliorate a symptom of such disorders in a mammalian subject, e.g. a cat.

15 Such disorders include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber
20 Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; wound granulation; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; diabetic neovascularization; macular degeneration; fractures; vasculogenesis; hematopoiesis; ovulation; menstruation; and placentation.

25 The invention further relates to methods for modulation of angiostatin-mediated processes and for the treatment of disorders involving abnormal stimulation of endothelial cells, including the amelioration or prevention of at least one symptom of the disorders, wherein such methods comprise administering a compound which modulates the expression of an angiostatin gene and/or the synthesis or activity of an angiostatin gene product. In one
30 embodiment, the invention relates to methods for the use of a novel angiostatin gene product or fragment, analog, or mimetic thereof, or an antibody or antibody fragment directed against an angiostatin gene product, to treat or ameliorate a symptom of such disorders in a mammalian subject, e.g. a cat.

35 The endothelial cell proliferation inhibiting proteins of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochelle minalia quintosa*) and ulcers (*Helicobacter pylori*).

The invention further relates to methods for blocking interactions between angiostatin and its respective receptor with analogs that act as receptor antagonists. These antagonists may promote endothelialization and vascularization. Such effects may be desirable in situations including, but not limited to, inadequate vascularization of the uterine endometrium and associated infertility, wound repair, healing of cuts and incisions, treatment of vascular problems in diabetics, especially retinal and peripheral vessels, promotion of vascularization in transplanted tissue including muscle and skin, promotion of vascularization of cardiac muscle especially following transplantation of a heart or heart tissue and after bypass surgery, promotion of vascularization of solid and relatively avascular tumors for enhanced cytotoxin delivery, and enhancement of blood flow to the nervous system, including but not limited to the cerebral cortex and spinal cord.

The term "angiostatin-related disorder" as used herein, refers to disorders involving an angiostatin gene or gene product, or an aberrant level of angiostatin gene expression, gene product synthesis and/or gene product activity, respectively, relative to levels found in normal, unaffected, unimpaired individuals, levels found in clinically normal individuals, and/or levels found in a population whose levels represent baseline, average angiostatin levels.

The term "angiostatin-mediated process" as used herein, includes processes dependent and/or responsive, either directly or indirectly, to the level of expression, gene product synthesis and/or gene product activity of angiostatin genes.

In another embodiment, such methods can comprise modulating the level of expression or the activity of an angiostatin gene product in a cell such that the angiostatin-mediated process or the disorder is treated, e.g., a symptom is ameliorated. In another embodiment, such methods can comprise supplying a nucleic acid molecule encoding an angiostatin gene product to increase the level, expression or activity of the angiostatin gene product within the cell such that the angiostatin-mediated process or the disorder is treated, e.g., a symptom is ameliorated. The nucleic acid molecule encoding the angiostatin gene product can encode a mutant angiostatin gene product with increased activity or expression levels.

The invention still further relates to methods for modulation of angiostatin-mediated processes or the treatment of angiostatin-related disorders, such as cancer, including, but not limited to, disorders resulting from angiostatin gene mutations, and/or an abnormal levels of angiostatin expression or activity and disorders involving one or more angiostatin genes or gene products, wherein treatment includes the amelioration or prevention of at least one symptom of such disorders in a mammalian subject, e.g. a cat. In one embodiment, such methods can comprise supplying a mammal (e.g. a cat) in need of treatment with a nucleic acid molecule encoding an unimpaired angiostatin gene product such that the unimpaired angiostatin gene product is expressed and the disorder is treated, e.g., a symptom is ameliorated. In another embodiment, such methods can comprise supplying a mammal (e.g. a cat) in need of treatment with a cell comprising a nucleic acid molecule that encodes an unimpaired angiostatin gene product such that the cell expresses the unimpaired angiostatin

gene product and the disorder is treated, *e.g.*, a symptom is ameliorated. In yet another embodiment, such methods comprise supplying a mammal in need of treatment with a modulatory compound, such as, for example, a small molecule, peptide or antibody that is capable of modulating the activity of an angiostatin gene or gene product.

5 In addition, the present invention is directed to methods that utilize angiostatin gene sequences and/or angiostatin gene product sequences for the diagnostic evaluation, genetic testing and/or prognosis of angiogenesis-related disorders, such as cancer. For example, the invention relates to methods for diagnosing angiogenesis-related disorders, *e.g.*, cancer, wherein such methods can comprise measuring angiostatin gene expression in a patient
10 sample of a mammalian subject (*e.g.* a cat), or detecting an angiostatin mutation that correlates with the presence or development of such a disorder, in the genome of a mammal (*e.g.* a cat) suspected of exhibiting such a disorder.

The present invention also is directed to utilizing the angiostatin gene sequences and/or gene products as markers for mapping of the human chromosome.

15 The invention still further relates to methods for identifying compounds capable of modulating the expression of an angiostatin gene and/or the synthesis or activity of an angiostatin gene product, wherein such methods comprise contacting a compound with a cell that expresses such an angiostatin gene, measuring the levels of angiostatin gene expression, gene product expression or gene product activity, and comparing such levels to
20 the levels of angiostatin gene expression, gene product, or gene product activity produced by the cell in the absence of such compound, such that if the level obtained in the presence of the compound differs from that obtained in its absence, a compound capable of modulating the expression of the angiostatin gene and/or the synthesis or activity of the angiostatin gene product has been identified.

25

ABBREVIATIONS

As used herein, the following terms shall have the abbreviations indicated.

	BAC:	bacterial artificial chromosome
	bp:	base pair(s)
30	dbEST:	expressed sequence tag data base (National Center for Biotechnology Information)
	EST:	expressed sequence tag
	RT-PCR:	reverse transcriptase PCR
	SSCP:	single-stranded conformational polymorphism
35	SNP:	single nucleotide polymorphism
	YAC:	yeast artificial chromosome

BRIEF DESCRIPTION OF THE FIGURES

- 40 Fig. 1: The nucleotide sequence of feline pro-angiostatin (SEQ ID NO: 1).
Fig. 2: The amino acid sequence of feline pro-angiostatin translated from the

sequence of Fig. 6 (SEQ ID NO: 2). The region corresponding to angiostatin is in bold (amino acid residues 38-401).

Fig. 3: The nucleotide sequence of feline angiostatin (SEQ ID NO: 3).

Fig. 4: The amino acid sequence of feline angiostatin translated from the sequence of Fig. 3 (SEQ ID NO: 4).

Fig. 5: An amino acid alignment of angiostatin from feline, human, mouse and bovine. The program used is Lasergene MegAlign, aligned by Clustal method (DNA Star Inc., Madison, WI).

Fig. 6: Immunoblot analysis of 293 cells transfected with plasmids expressing HA-tagged versions of feline angiostatin (Fe-Angio) and HA-tagged murine angiostatin (Mu-Angio). Secreted proteins from culture supernatants were separated by SDS-PAGE and detected by Western blotting using antibody directed against HA-tag and alkaline phosphatase conjugated secondary antibody.

Fig. 7: Immunoblot analysis of 293 cells transfected with plasmids expressing HA-tagged versions of feline angiostatin (Fe-Angio) and HA-tagged murine angiostatin (Mu-Angio). Intracellular proteins from total cell lysates were separated by SDS-PAGE and detected by Western blotting using antibody directed against HA-tag and alkaline phosphatase conjugated secondary antibody.

Fig. 8: Endothelial Cell Proliferation Assay. The figure shows a summary of inhibition of endothelial cell proliferation by HA-tagged feline angiostatin (HA-fe-angio) as well as murine angiostatin (HA-mu-angio). 293 cells were transfected with angiogenesis inhibitors or green fluorescent protein (GFP) as control. The supernatants were harvested 48 hours post transfection and incubated with bFGF stimulated CPAE bovine endothelial cells or 293 cells for 72 hours. The total numbers of cells were counted and plotted. Four independent experiments were carried out and each experiment was done in duplicate. Filled bars represent percent of stimulated CPAE cells, while open bars represent percent of stimulated 293 cells.

Fig. 9: Endothelial Cell Proliferation Assay. The figure shows a summary of inhibition of endothelial cell proliferation by an untagged version of feline angiostatin (fe-angio). 293 cells were transfected with angiogenesis inhibitors or green fluorescent protein (GFP) as control. The supernatants were harvested 48 hours post transfection and incubated with bFGF stimulated CPAE bovine endothelial cells or 293 cells for 72 hours. The total numbers of cells were counted and plotted. Four independent experiments were carried out and each experiment was done in duplicate. Filled and open bars are as described above.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods relating to nucleic acid sequences associated with disorders involving angiogenesis are described herein. Novel genes which are associated with angiogenesis-related disorders have been identified. Such genes encode angiostatin. In particular, described below are angiostatin nucleic acid molecules, as well as vectors

comprising these molecules, host cells engineered to contain and/or express such molecules, angiostatin gene products, and antibodies that specifically recognize such gene products. Also described are various uses of these nucleic acids, polypeptides, and antibodies, as well as methods for their detection. For example, methods for the use of these molecules for modulation of angiogenesis-related processes and for treatment of angiogenesis-related disorders, such as cancer, are described. Assays for screening compounds that interact with an angiostatin gene or gene product, or modulate angiostatin gene or gene product activity also are described below. Methods of treatment of an angiogenesis-related disorder using the compositions of the invention and compositions identified by the methods of the invention are further described. Finally, pharmaceutical compositions for use with the compositions of the invention are described.

The term "animal or subject" or "subject" as used herein refers to any mammalian subject, e.g. human, mouse, and cat. The compositions and methods provided by the present invention are particularly useful for application to cats.

Angiostatin nucleic acid molecules are described in this section. Unless otherwise stated, the term "angiostatin nucleic acid" refers collectively to the sequences described herein.

The angiostatin nucleic acid molecules of the invention include:

- (a) a nucleic acid molecule containing the DNA sequence of angiostatin (Fig. 3 (SEQ ID NO:3)), and fragments thereof;
- (b) a nucleic acid molecule comprising an angiostatin nucleic acid sequence (e.g., the nucleic acid sequences depicted in Fig. 1 (SEQ ID NO:1) or a fragment thereof;
- (c) a nucleic acid molecule that encodes an angiostatin gene product (e.g. an angiostatin polypeptide);
- (d) a nucleic acid molecule that comprises at least one exon of an angiostatin gene;
- (e) a nucleic acid molecule that comprises angiostatin gene sequences of upstream untranslated regions, intronic regions, and/or downstream untranslated regions, or fragments thereof, of the angiostatin nucleotide sequences in (b) above;
- (f) a nucleic acid molecule that encodes a mutant of an angiostatin gene product in which all or a part of one or more of the domains is deleted or altered, as well as fragments thereof;
- (g) nucleic acid molecules that encode fusion proteins comprising an angiostatin gene product, or a fragment thereof, fused to a heterologous polypeptide;
- (h) nucleic acid molecules within the angiostatin genes described in b), above (e.g., primers), or within chromosomal nucleotide sequences flanking the angiostatin gene, which can be utilized as part of the methods of the invention for identifying and diagnosing individuals at risk for, or exhibiting an angiogenesis-related disorder, such as cancer, or can be used for mapping human chromosomes; and;

(i) nucleic acid molecules within the angiostatin genes described in b), above, or within chromosomal nucleotide sequences flanking the angiostatin, which correlate with an angiogenesis-related disorder, such as cancer.

5 The angiostatin nucleotide sequences of the invention further include nucleotide sequences corresponding to the nucleotide sequences of (a)-(i) above wherein one or more of the exons, or fragments thereof, have been deleted.

The angiostatin nucleotide sequences of the invention also include nucleotide sequences greater than 20, 30, 40, 50, 60, 70, 80, 90, 100, or more base pairs long that have at least 85%, 90%, 95%, 98%, or more nucleotide sequence identity to the angiostatin
10 nucleotide sequences of (a)-(i) above, with the proviso that the angiostatin is not human, canine, mouse or bovine angiostatin.

The angiostatin nucleotide sequences of the invention further include nucleotide sequences that encode polypeptides having at least 85%, 90%, 95%, 98%, or higher amino acid sequence identity to the polypeptides encoded by the angiostatin nucleotide sequences of
15 (a)-(i) above.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or
20 nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = # of identical
25 overlapping positions/total # of overlapping positions x 100%). In one embodiment, the two sequences are of the same length.

The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and
30 Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of
35 the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships
40 between molecules (Altschul et al., 1997, *supra*). When utilizing BLAST, Gapped BLAST, and

PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used (see <http://www.ncbi.nlm.nih.gov>). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul et al., 1997, *supra*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used (see <http://www.ncbi.nlm.nih.gov>). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

The angiostatin nucleotide sequences of the invention further include: (a) any nucleotide sequence that hybridizes to an angiostatin nucleic acid molecule of the invention under moderately stringent conditions, e.g., hybridization to filter-bound DNA in 6x sodium chloride/sodium citrate (SSC) at about 45°C followed by one or more washes in 0.2xSSC/0.1% SDS at about 50-65°C, or (b) under highly stringent conditions, e.g., hybridization to filter-bound nucleic acid in 6xSSC at about 45°C followed by one or more washes in 0.1x SSC/0.2% SDS at about 68°C, or under other hybridization conditions which are apparent to those of skill in the art (see, for example, Ausubel F.M. et al., eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at pp. 6.3.1-6.3.6 and 2.10.3). Preferably the angiostatin nucleic acid molecule that hybridizes under conditions described under (a) and (b), above, is one that comprises the complement of a nucleic acid molecule that encodes an angiostatin gene product. In a preferred embodiment, nucleic acid molecules that hybridize under

conditions (a) and (b), above, encode gene products, e.g., gene products functionally equivalent to an angiostatin gene product.

Functionally equivalent angiostatin gene products include naturally occurring angiostatin gene products present in the same or different species. Functionally equivalent
 5 angiostatin gene products also include gene products that retain at least one of the biological activities of an angiostatin gene product, and/or which are recognized by and bind to antibodies (polyclonal or monoclonal) directed against such gene product.

Among the nucleic acid molecules of the invention are deoxyoligonucleotides ("oligos") which hybridize under highly stringent or stringent conditions to the angiostatin
 10 nucleic acid molecules described above. In general, for probes between 14 and 70 nucleotides in length the melting temperature (T_m) is calculated using the formula: $T_m(^{\circ}\text{C}) = 81.5 + 16.6 (\log [\text{monovalent cations (molar)}]) + 0.41 (\% \text{ G+C}) - (500/N)$ where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation $T_m(^{\circ}\text{C}) = 81.5 + 16.6 (\log [\text{monovalent cations (molar)}]) + 0.41 (\% \text{ G+C}) - (0.61\% \text{ formamide}) - (500/N)$ where N is the length of the probe. In
 15 general, hybridization is carried out at about 20-25 degrees below T_m (for DNA-DNA hybrids) or 10-15 degrees below T_m (for RNA-DNA hybrids).

Exemplary highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for about 14-base oligos), 48°C (for about 17-base oligos),
 20 55°C (for about 20-base oligos), and 60°C (for about 23-base oligos).

The nucleic acid molecules of the invention further comprise the complements of the nucleic acids described above. Such molecules can, for example, act as antisense molecules, useful, for example, in angiostatin gene regulation, and/or as antisense primers in amplification reactions of angiostatin gene nucleic acid sequences.

25 The nucleic acid sequences of the invention may be used as part of ribozyme and/or triple helix sequences, also useful for angiostatin gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby, for example, the presence of a particular angiostatin allele involved in an angiogenesis-related disorder, e.g., cancer, may be detected, or whereby the methods involve mapping the human chromosomal region spanned
 30 by the alleles.

Fragments of the angiostatin nucleic acid molecules refer to angiostatin nucleic acid sequences that can be at least 10, 12, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500, 4750, 5000, or more contiguous nucleotides in length. Alternatively,
 35 the fragments can comprise sequences that encode at least 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450 or more contiguous amino acid residues of the angiostatin gene products. In one embodiment, the angiostatin nucleic acid molecules encode a gene product exhibiting at least one biological activity of a corresponding angiostatin gene product, e.g., an angiostatin gene product. Fragments of the angiostatin nucleic acid molecules can refer also

to angiotatin exons or introns, and, further, can refer to portions of angiotatin coding regions that encode domains of angiotatin gene products.

With respect to identification and isolation of angiotatin nucleotide sequences, such sequences can be readily obtained, for example, by utilizing standard sequencing and bacterial artificial chromosome (BAC) technologies.

As will be appreciated by those skilled in the art, DNA sequence polymorphisms of an angiotatin gene will exist within a population of individual organisms (*e.g.*, within a human or feline population). Such polymorphisms may exist, for example, among individual organisms within a population due to natural allelic variation. Such polymorphisms include ones that lead to changes in amino acid sequence. As used herein, the phrase "allelic variant" refers to a nucleotide sequence which occurs at a given locus or to a gene product encoded by that nucleotide sequence. Such natural allelic variations can result in 1-5%, 5-20%, or 20-50% variance in the nucleotide sequence of a given gene. An allele is one of a group of genes which occur alternatively at a given genetic locus. Alternative alleles can be identified by sequencing the gene of interest in a number of different individual organisms. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individual organisms. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide of the invention. The term can further include nucleic acid molecules comprising upstream and/or exon/intron sequences and structure.

With respect to the cloning of additional allelic variants of the human angiotatin gene and homologs and orthologs from other species (*e.g.*, guinea pig, cow, mouse, feline), the isolated angiotatin gene sequences disclosed herein may be labeled and used to screen a cDNA library constructed from mRNA obtained from appropriate cells or tissues (*e.g.*, liver) derived from the organism (*e.g.*, guinea pig, cow, mouse, feline) of interest. The hybridization conditions used should generally be of a lower stringency when the cDNA library is derived from an organism different from the type of organism from which the labeled sequence was derived, and can routinely be determined based on, *e.g.*, relative relatedness of the target and reference organisms.

Alternatively, the labeled fragment may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. Appropriate stringency conditions are well known to those of skill in the art as discussed above, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook, *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, N.Y.; and Ausubel, *et al.*, 1989-1999, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y., both of which are incorporated herein by reference in their entirety.

Further, an angiotatin gene allelic variant may be isolated from, for example, human or feline nucleic acid, by performing PCR using two degenerate oligonucleotide primer pools

designed on the basis of amino acid sequences within the angiostatin gene products disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue known or suspected to express a wild type or mutant angiostatin gene allele (such as, for example, liver cells). In one embodiment, the allelic variant is isolated from an individual organism that has an angiogenesis-mediated disorder.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of an angiostatin gene nucleic acid sequence. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology also may be utilized to isolate full length cDNA sequences, as well as cDNA sequences corresponding to alternatively spliced mRNA species. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source (*i.e.*, one known, or suspected, to express the angiostatin gene, such as, for example, liver tissue samples obtained through biopsy or post-mortem). A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies that may be used, see *e.g.*, Sambrook *et al.*, 1989, *supra*, or Ausubel *et al.*, *supra*.

A cDNA of an allelic, *e.g.*, mutant, variant of the angiostatin gene may be isolated, for example, by using PCR, a technique that is well known to those of skill in the art. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual organism putatively carrying a mutant angiostatin allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant angiostatin allele to that of the normal angiostatin allele, the mutation(s) responsible for the loss or alteration of function of the mutant angiostatin gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from an individual organism suspected of or known to carry a mutant angiostatin allele, or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant angiostatin allele. An unimpaired angiostatin gene, or any suitable fragment thereof, may then

be labeled and used as a probe to identify the corresponding mutant angiostatin allele in such libraries. Clones containing the mutant angiostatin gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

5 Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant angiostatin allele in an individual organism suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal angiostatin gene product, as described, below. (For screening techniques, see, for
10 example, Harlow and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

In cases where an angiostatin mutation results in an expressed gene product with altered function (e.g., as a result of a missense or a frameshift mutation), a polyclonal set of anti-angiostatin gene product antibodies are likely to cross-react with the mutant angiostatin
15 gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Angiostatin mutations or polymorphisms can further be detected using PCR amplification techniques. Primers can routinely be designed to amplify overlapping regions of
20 the whole angiostatin sequence including the promoter regulating region. In one embodiment, primers are designed to cover the exon-intron boundaries such that, coding regions can be scanned for mutations.

The invention also includes nucleic acid molecules, preferably DNA molecules, that are the complements of the nucleotide sequences of the preceding paragraphs.

25 In certain embodiments, the nucleic acid molecules of the invention are present as part of nucleic acid molecules comprising nucleic acid sequences that contain or encode heterologous (e.g., vector, expression vector, or fusion protein) sequences.

Angiostatin gene products include those gene products encoded by nucleic acid molecules comprising the angiostatin gene sequences described, above. In addition,
30 angiostatin gene products may include proteins that represent functionally equivalent gene products. Such an equivalent angiostatin gene product may contain deletions, including internal deletions, additions, including additions yielding fusion proteins, or substitutions of amino acid residues within and/or adjacent to the amino acid sequence encoded by the angiostatin gene sequences described, above, but that result in a "silent" change, in that the
35 change produces a functionally equivalent angiostatin gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine,
40 cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include

arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Alternatively, where alteration of function is desired, deletion or non-conservative alterations can be engineered to produce altered angiotensin gene products. Such alterations can, for example, alter one or more of the biological functions of the angiotensin gene product. Further, such alterations can be selected so as to generate angiotensin gene products that are better suited for expression, scale up, etc. in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges.

Peptides and/or proteins corresponding to one or more domains of an angiotensin protein, as well as fusion proteins, in which an angiotensin protein or a portion of an angiotensin protein, such as a truncated angiotensin protein, or peptide or an angiotensin protein domain, is fused to an unrelated protein are also within the scope of this invention. Such proteins and peptides can be designed on the basis of the angiotensin nucleotide sequence disclosed, above, and/or on the basis of the angiotensin amino acid sequence disclosed herein. Fusion proteins include, but are not limited to, IgFc fusions which stabilize the angiotensin protein or peptide and prolong half life *in vivo*; or fusions to any amino acid sequence that allows the fusion protein to be anchored to the cell membrane; or fusions of angiotensin protein domains to an enzyme, fluorescent protein, luminescent protein, or a flag epitope protein or peptide which provides a marker function.

Angiotensin proteins of the invention also include angiotensin protein sequences wherein domains encoded by at least one exon of the cDNA sequence, or fragments thereof, have been deleted.

The angiotensin protein sequences described above can include a domain which comprises a signal sequence that targets the angiotensin gene product for secretion. As used herein, a signal sequence includes a peptide of at least about 15 or 20 amino acid residues in length which occurs at the N-terminus of secretory and membrane-bound proteins and which contains at least about 70% hydrophobic amino acid residues such as alanine, leucine, isoleucine, phenylalanine, proline, tyrosine, tryptophan, or valine. In a preferred embodiment, a signal sequence contains at least about 10 to 40 amino acid residues, preferably about 19-34 amino acid residues, and has at least about 60-80%, more preferably 65-75%, and more preferably at least about 70% hydrophobic residues. A signal sequence serves to direct a protein containing such a sequence to a lipid bilayer.

A signal sequence of a polypeptide of the invention can be used to facilitate secretion and isolation of the secreted protein or other proteins of interest. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. Thus, the invention pertains to the described angiotensin polypeptides having a signal sequence (that is, "immature" polypeptides), as well

as to the angiotensin signal sequences themselves and to the angiotensin polypeptides in the absence of a signal sequence (*i.e.*, the "mature" angiotensin cleavage products). It is to be understood that angiotensin polypeptides of the invention can further comprise polypeptides comprising any signal sequence having characteristics as described above and a mature angiotensin polypeptide sequence.

The angiotensin polypeptides of the invention can further comprise posttranslational modifications, including, but not limited to, glycosylations, acetylations, myristylations, and phosphorylations. If the native angiotensin protein does not have recognition motifs that allow such modifications, it would be routine for one skilled in the art to introduce into an angiotensin gene nucleotide sequences that encode motifs such as enzyme recognition signals so as to produce a modified angiotensin gene product.

The angiotensin gene products, peptide fragments thereof and fusion proteins thereof, may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the angiotensin gene polypeptides, peptides, fusion peptide and fusion polypeptides of the invention by expressing nucleic acid containing angiotensin gene sequences are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing angiotensin gene product coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, *e.g.*, the techniques described in Sambrook, *et al.*, 1989, *supra*, and Ausubel, *et al.*, 1989, *supra*. Alternatively, RNA capable of encoding angiotensin gene product sequences may be chemically synthesized using, for example, synthesizers. See, *e.g.*, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, ed., IRL Press, Oxford.

A variety of host-expression vector systems may be utilized to express the angiotensin gene coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the angiotensin gene product of the invention *in situ*. These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing angiotensin gene product coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the angiotensin gene product coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the angiotensin gene product coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing angiotensin gene product coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*,

metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the angiotensin gene product being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of angiotensin protein or for raising antibodies to angiotensin protein, for example, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, *EMBO J.* 2, 1791), in which the angiotensin gene product coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye and Inouye, 1985, *Nucleic Acids Res.* 13, 3101-3109; Van Heeke and Schuster, 1989, *J. Biol. Chem.* 264, 5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. Angiotensin gene coding sequences may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of angiotensin gene coding sequences will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith, *et al.*, 1983, *J. Virol.* 46, 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, an angiotensin gene coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing angiotensin gene product in infected hosts. (e.g., See Logan and Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81, 3655-3659). Specific initiation signals may also be required for efficient translation of inserted angiotensin gene product coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire angiotensin gene, including an initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals

may be needed. In cases where only a portion of the angiotatin gene coding sequence is inserted, similar exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These
5 exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner, *et al.*, 1987, *Methods in Enzymol.* 153, 516-544).

In addition, a host cell strain may be chosen that modulates the expression of the
10 inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure
15 the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the angiotatin gene product may be engineered. Rather than using expression vectors that contain viral origins of replication, host
20 cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines. This method may advantageously be
25 used to engineer cell lines that express the angiotatin gene product. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the angiotatin gene product.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (Wigler, *et al.*, 1977, *Cell* 11, 223), hypoxanthine-guanine
35 phosphoribosyltransferase (Szybalska and Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48, 2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, *Cell* 22, 817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler, *et al.*, 1980, *Proc. Natl. Acad. Sci. USA* 77, 3567; O'Hare, *et al.*, 1981,
40 *Proc. Natl. Acad. Sci. USA* 78, 1527); gpt, which confers resistance to mycophenolic acid

(Mulligan and Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78, 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, *J. Mol. Biol.* 150, 1); and hygro, which confers resistance to hygromycin (Santerre, *et al.*, 1984, *Gene* 30, 147).

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht, *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88, 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} -nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Alternatively, the expression characteristics of an endogenous angiostatin gene within a cell, cell line, or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous angiostatin gene. For example, an endogenous angiostatin gene which is normally "transcriptionally silent", *i.e.*, an angiostatin gene which is normally not expressed, or is expressed only at very low levels in a cell, cell line, or microorganism, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed gene product in that cell, cell line, or microorganism. Alternatively, a transcriptionally silent, endogenous angiostatin gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such that it is operatively linked with an endogenous angiostatin gene, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art, and described *e.g.*, in Chappel, U.S. Patent No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991.

Angiostatin gene products also can be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, sheep, and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate angiostatin transgenic animals. The term "transgenic," as used herein, refers to animals expressing angiostatin gene sequences from a different species (*e.g.*, mice expressing human or feline angiostatin sequences), as well as animals that have been genetically engineered to overexpress endogenous (*i.e.*, same species) angiostatin sequences or animals that have been genetically engineered to no longer express endogenous angiostatin gene sequences (*i.e.*, "knock-out" animals), and their progeny.

Any technique known in the art may be used to introduce an angiostatin gene transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Hoppe and Wagner, 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (van der Putten, *et al.*, 1985,

Proc. Natl. Acad. Sci., USA 82, 6148-6152); gene targeting in embryonic stem cells (Thompson, *et al.*, 1989, *Cell* 56, 313-321); electroporation of embryos (Lo, 1983, *Mol. Cell. Biol.* 3, 1803-1814); and sperm-mediated gene transfer (Lavitrano *et al.*, 1989, *Cell* 57, 717-723). (For a review of such techniques, see Gordon, 1989, *Transgenic Animals, Intl. Rev. Cytol.* 115, 171-229.)

Any technique known in the art may be used to produce transgenic animal clones containing an angiostatin transgene, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal or adult cells induced to quiescence (Campbell, *et al.*, 1996, *Nature* 380, 64-66; Wilmut, *et al.*, *Nature* 385, 810-813).

The present invention provides for transgenic animals that carry an angiostatin transgene in all their cells, as well as animals that carry the transgene in some, but not all their cells, *i.e.*, mosaic animals. The transgene may be integrated as a single transgene or in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (1992, *Proc. Natl. Acad. Sci. USA* 89, 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the angiostatin gene transgene be integrated into the chromosomal site of the endogenous angiostatin gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous angiostatin gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous angiostatin gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous angiostatin gene in only that cell type, by following, for example, the teaching of Gu, *et al.* (1994, *Science* 265, 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the phenotypic expression of the recombinant angiostatin gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques that include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR (reverse transcriptase PCR). Samples of angiostatin gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the angiostatin transgene product.

Angiostatin gene products, or peptide fragments thereof, can be prepared for a variety of uses. For example, such gene products, or peptide fragments thereof, can be used for the generation of antibodies, in diagnostic assays, or for mapping and the identification of other cellular or extracellular gene products involved in the regulation of an angiogenesis-related

disorder, such as cancer. Such angiostatin gene products include, but are not limited to, soluble derivatives such as peptides or polypeptides corresponding to one or more domains of the angiostatin gene product, particularly angiostatin gene products that are modified such that they are deleted for one or more hydrophobic domains. Alternatively, antibodies to the angiostatin protein or anti-Idiotypic antibodies that mimic the angiostatin gene product (including Fab fragments), antagonists or agonists can be used to treat angiogenesis-related disorders, such as cancer. In yet another approach, nucleotide constructs encoding such angiostatin gene products can be used to genetically engineer host cells to express such angiostatin gene products *in vivo*; these genetically engineered cells can function as "bioreactors" in the body delivering a continuous supply of angiostatin gene product, angiostatin peptides or soluble angiostatin polypeptides.

Described herein are methods for the production of antibodies capable of specifically recognizing one or more angiostatin gene product epitopes or epitopes of conserved variants or peptide fragments of the gene products.

Such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), feline, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-Idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of an angiostatin gene product in a biological sample and may, therefore, be utilized as part of a diagnostic or prognostic technique whereby subjects may be tested for abnormal levels of angiostatin gene products, and/or for the presence of abnormal forms of such gene products. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described below, for the evaluation of the effect of test compounds on angiostatin gene product levels and/or activity. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described below, for example, to evaluate the normal and/or engineered angiostatin-expressing cells prior to their introduction into the subject.

Anti-angiostatin gene product antibodies may additionally be used as a method for the inhibition of abnormal angiostatin gene product activity. Thus, such antibodies may be utilized as part of treatment methods for an angiogenesis-related disorder, *e.g.*, cancer.

For the production of antibodies against an angiostatin gene product, various host animals may be immunized by injection with an angiostatin gene product, or a portion thereof. Such host animals may include, but are not limited to, rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as an angiostatin gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with angiostatin gene product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256, 495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4, 72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80, 2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. (See, *e.g.*, Cabilly *et al.*, U.S. Patent No. 4,816,567; and Boss *et al.*, U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, *e.g.*, Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.) Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Bio/Techniques* 4:214; U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Such antibodies can be produced, for example, using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized
5 in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce
10 therapeutically useful IgG, IgA and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S.
15 Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Fremont, CA), can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human
20 monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespersen et al. (1994) *Bio/technology* 12:899-903).

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, *Science* 242, 423-426; Huston, et al., 1988, *Proc. Natl. Acad. Sci. USA* 85, 5879-5883; and Ward, et al., 1989, *Nature* 334, 544-546) can be adapted to
25 produce single chain antibodies against angiotensin gene products. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known
30 techniques. For example, such fragments include, but are not limited to, the F(ab')₂ fragments, which can be produced by pepsin digestion of the antibody molecule and the Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse, et al., 1989, *Science*, 246, 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the
35 desired specificity.

Described herein are various applications of angiotensin gene sequences, angiotensin gene products, including peptide fragments and fusion proteins thereof, and of antibodies directed against angiotensin gene products and peptide fragments thereof. Such applications include, for example, prognostic and diagnostic evaluation of an angiogenesis-related
40 disorder, e.g., cancer, and the identification of subjects with a predisposition to such disorders,

as described, below, in Section. Additionally, such applications include methods for the identification of compounds that modulate the expression of an angiostatin gene and/or the synthesis or activity of an angiostatin gene product, as described below, and for the treatment of an angiogenesis-related disorder, *e.g.* cancer, as described, below.

5 In addition, angiostatin gene sequences and gene products, including peptide fragments and fusion proteins thereof, and antibodies directed against angiostatin gene products and peptide fragments thereof, have applications for purposes independent of the role angiostatin may have in angiogenesis-related disorders and processes. For example, angiostatin gene products, including peptide fragments, as well as angiostatin-specific
10 antibodies, can be used for construction of fusion proteins to facilitate recovery, detection, or localization of another protein of interest. In addition, angiostatin genes and gene products can be used for genetic mapping, *i.e.*, refining the genetic map. For example, antibodies specific to angiostatin can be used as probes to detect expression of human angiostatin in somatic cell hybrids containing human chromosomes, or portions of human chromosomes.
15 Such angiostatin-specific antibodies can be used to identify cells that contain the angiostatin chromosomal region. This method can be used, for example, to localize a gene or trait of interest to this region of the chromosome.

 Angiostatin gene sequences and gene products can be used for mapping and refining a chromosomal map. The angiostatin sequence can be used to develop new genetic markers
20 to further refine chromosomal intervals that are associated with various angiogenesis-related disorders, including, but not limited to, cancer. As will be apparent to the skilled artisan, nucleic acid sequences within a genetic interval associated with a disease can be scanned for new markers, such as microsatellites. Microsatellites, also known as simple-sequence repeats (SSRs), are hypervariable tandem-sequence repeats consisting of di-, tri-, or
25 tetranucleotide repeats of 1-5 nucleotides. Such microsatellites make excellent genetic markers for linkage studies since they are distributed ubiquitously throughout the human genome, are highly variable in repeat length, and tend to be highly polymorphic. Relatively common microsatellites (*e.g.*, (CA)_n dinucleotide repeats) occur approximately every 300-500 kb. In addition to microsatellite repeats, the region can be scanned for other types of
30 polymorphic sites useful for fine mapping, such as minisatellites (9-64 nucleotide repeats), restriction fragment length polymorphisms (RFLPs), and single nucleotide polymorphisms, which occur much less frequently. Once a polymorphic site is identified in a new sequence, PCR primers that flank the polymorphic site can be synthesized and used to amplify the microsatellite or other polymorphic site. The length of the repeat can then be determined by
35 resolving the PCR product on a polyacrylamide sequencing gel. Genomic DNA from human populations can then be analyzed for the simple-sequence length polymorphisms (SSLPs) to determine the frequency and variability of the repeat. Once a high quality SSLP is found, linkage analysis can be performed on an affected population to determine whether an angiogenesis-related disorder, such as cancer, is linked to the new marker. Other techniques,
40 such as Southern blot hybridization and ligase-chain reaction (LCR), can be used in addition

to, or in conjunction with, PCR-based methods to analyze polymorphisms in genomic populations (see, Current Protocols in Human Genetics, Dracopoli et al. (eds.) John Wiley & Sons, 1998).

5 In another embodiment, an angiotensin gene, protein or a fragment or domain thereof, can be used for construction of fusion proteins. Finally, angiotensin nucleic acids and gene products have generic uses, such as supplemental sources of nucleic acids, proteins and amino acids for food additives or cosmetic products.

10 Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) screen for angiotensin gene-specific mutations or polymorphisms, (ii) map their respective genes on a chromosome and, thus, locate gene regions associated with genetic disease; (iii) identify an individual organism from a minute biological sample (tissue typing); and (iv) aid in forensic identification of a biological sample. These applications are described in the subsections below.

15 A variety of methods can be employed to screen for the presence of angiotensin gene-specific mutations or polymorphisms (including polymorphisms flanking an angiotensin gene, e.g., ones that cosegregate with a particular angiotensin allele) and to detect and/or assay levels of angiotensin nucleic acid sequences.

20 Mutations or polymorphisms within or flanking the angiotensin gene can be detected by utilizing a number of techniques. Nucleic acid from any nucleated cell, or any cell that expresses the angiotensin gene of interest, can be used as the starting point for such assay techniques, and may be isolated according to standard nucleic acid preparation procedures that are well known to those of skill in the art.

25 Angiotensin nucleic acid sequences may be used in hybridization or amplification assays of biological samples to detect abnormalities involving angiotensin gene structure, including point mutations, insertions, deletions, inversions, translocations and chromosomal rearrangements. Such assays may include, but are not limited to, Southern analyses, single-stranded conformational polymorphism analyses (SSCP), and PCR analyses.

30 Diagnostic methods for the detection of angiotensin gene-specific mutations or polymorphisms can involve, for example, contacting and incubating nucleic acids obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source with one or more labeled nucleic acid reagents including recombinant DNA molecules, cloned genes or degenerate variants thereof, such as described, above, under conditions favorable for the specific annealing of these reagents to their complementary sequences within or flanking the angiotensin gene. The diagnostic methods of the present invention further encompass contacting and incubating nucleic acids for the detection of single nucleotide mutations or polymorphisms of the angiotensin gene. Preferably, these nucleic acid reagent sequences within the angiotensin gene, or chromosome nucleotide sequences flanking the angiotensin gene are 15 to 30 nucleotides in length.

After incubation, all non-annealed nucleic acids are removed from the nucleic acid:angiostatin molecule hybrid. The presence of nucleic acids that have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the cell type or tissue of interest can be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtiter plate or polystyrene beads. In this case, after incubation, non-annealed, labeled nucleic acid reagents of the type described above, are easily removed. Detection of the remaining, annealed, labeled angiostatin nucleic acid reagents is accomplished using standard techniques well-known to those in the art. The angiostatin gene sequences to which the nucleic acid reagents have annealed can be compared to the annealing pattern expected from a normal angiostatin gene sequence in order to determine whether an angiostatin gene mutation is present.

In a preferred embodiment, angiostatin mutations or polymorphisms can be detected by using a microassay of angiostatin nucleic acid sequences immobilized to a substrate or "gene chip" (see, e.g. Cronin *et al.*, 1996, *Human Mutation* 7:244-255).

Alternative diagnostic methods for the detection of angiostatin gene-specific nucleic acid molecules (or angiostatin flanking sequences), in patient samples or other appropriate cell sources, may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, 1987, U.S. Patent No. 4,683,202), followed by the analysis of the amplified molecules using techniques well known to those of skill in the art, such as, for example, those listed above. The resulting amplified sequences can be compared to those that would be expected if the nucleic acid being amplified contained only normal copies of the angiostatin gene in order to determine whether an angiostatin gene mutation or polymorphism in linkage disequilibrium with a disease-causing angiostatin allele exists.

Additionally, well-known genotyping techniques can be performed to identify individual organisms carrying angiostatin gene mutations. Such techniques include, for example, the use of restriction fragment length polymorphisms (RFLPs), which involve sequence variations in one of the recognition sites for the specific restriction enzyme used.

Further, improved methods for analyzing DNA polymorphisms, which can be utilized for the identification of angiostatin gene-specific mutations, have been described that capitalize on the presence of variable numbers of short, tandemly repeated DNA sequences between the restriction enzyme sites. For example, Weber (U.S. Pat. No. 5,075,217) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)_n-(dG-dT)_n short tandem repeats. The average separation of (dC-dA)_n-(dG-dT)_n blocks is estimated to be 30,000-60,000 bp. Markers that are so closely spaced exhibit a high frequency co-inheritance, and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the angiostatin gene, and the diagnosis of diseases and disorders related to angiostatin mutations.

Also, Caskey *et al.* (U.S. Pat. No. 5,364,759) describe a DNA profiling assay for detecting short tri and tetra nucleotide repeat sequences. The process includes extracting the

DNA of interest, such as the angiostatin gene, amplifying the extracted DNA, and labeling the repeat sequences to form a genotypic map of the individual organism's DNA.

Other methods well known in the art may be used to identify single nucleotide polymorphisms (SNPs), including biallelic SNPs or biallelic markers which have two alleles, both of which are present at a fairly high frequency in a population. Conventional techniques for detecting SNPs include, *e.g.*, conventional dot blot analysis, SSCP analysis (see, *e.g.*, Orita *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:2766-2770), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, and other routine techniques well known in the art (see, *e.g.*, Sheffield *et al.*, 1989, *Proc. Natl. Acad. Sci.* 86:5855-5892; Grompe, 1993, *Nature Genetics* 5:111-117). Alternative, preferred methods of detecting and mapping SNPs involve microsequencing techniques wherein an SNP site in a target DNA is detected by a single nucleotide primer extension reaction (see, *e.g.*, Goelet *et al.*, PCT Publication No. WO92/15712; Mundy, U.S. Patent No. 4,656,127; Vary and Diamond, U.S. Patent No. 4,851,331; Cohen *et al.*, PCT Publication No. WO91/02087; Chee *et al.*, PCT Publication No. WO95/11995; Landegren *et al.*, 1988, *Science* 241:1077-1080; Nicerson *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:8923-8927; Pastinen *et al.*, 1997, *Genome Res.* 7:606-614; Pastinen *et al.*, 1996, *Clin. Chem.* 42:1391-1397; Jalanko *et al.*, 1992, *Clin. Chem.* 38:39-43; Shumaker *et al.*, 1996, *Hum. Mutation* 7:346-354; Caskey *et al.*, PCT Publication No. WO 95/00669).

The level of angiostatin expression also can be determined by first assaying for the level of gene expression. For example, RNA from a cell type or tissue known, or suspected, to express the angiostatin gene, such as liver, may be isolated and tested utilizing hybridization or PCR techniques such as are described, above. The isolated cells can be derived from cell culture or from a subject. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the angiostatin gene. Such analyses may reveal both quantitative and qualitative aspects of the expression pattern of the angiostatin gene, including activation or inactivation of angiostatin gene expression.

In one embodiment of such a detection scheme, a cDNA molecule is synthesized from an RNA molecule of interest (*e.g.*, by reverse transcription of the RNA molecule into cDNA). A sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (*e.g.*, primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the angiostatin gene nucleic acid reagents described above. The preferred lengths of such nucleic acid reagents are at least 9-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized

by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

Additionally, it is possible to perform such angiostatin gene expression assays *in situ*, *i.e.*, directly upon tissue sections (fixed and/or frozen) of subject tissue obtained from biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described above, may be used as probes and/or primers for such *in situ* procedures (see, for example, Nuovo, G.J., 1992, "PCR *In Situ* Hybridization: Protocols And Applications", Raven Press, NY).

Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern analysis can be performed to determine the level of mRNA expression of the angiostatin gene.

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. Accordingly, nucleic acid molecules described herein, or fragments thereof, can be used to map the location of the corresponding genes on a chromosome. The mapping of the sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the sequence of a gene of the invention. Computer analysis of the sequence of a gene of the invention can be used to rapidly select primers that do not span more than one exon in the genomic DNA, thus simplifying the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the gene sequences will yield an amplified fragment. For a review of this technique, see D'Eustachio et al. (1983, *Science*, 220:919-924).

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the nucleic acid sequences of the invention to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a gene to its chromosome include *in situ* hybridization (described in Fan et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:6223-27), pre-screening with labeled flow-sorted chromosomes (Popp S, et al., 1993, *Hum Genet.*, 92(6):527-32) and pre-selection by hybridization to chromosome specific cDNA libraries. Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. (For a review of this technique, see Verma et al., *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York, 1988.)

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding

regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

5 Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, e.g., Egeland et al. (1987, 10 *Nature* 325:783-787).

 Moreover, differences in the DNA sequences between individual organisms affected and unaffected with a disease associated with a gene of the invention can be determined. If a mutation is observed in some or all of the affected individual organisms but not in any unaffected individual organisms, then the mutation is likely to be the causative agent of the 15 particular disease. Comparison of affected and unaffected individual organisms generally involves first looking for structural alterations in the chromosomes such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individual organisms can be performed to confirm the presence of a mutation and to distinguish 20 mutations from polymorphisms.

 The nucleic acid sequences of the present invention also can be used to identify individual organisms from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual organism's genomic DNA is digested with one or 25 more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

30 Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual organism's genome. Thus, the nucleic acid sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual organism's DNA and subsequently 35 sequence it.

 Panels of corresponding DNA sequences from individual organisms, prepared in this manner, can provide unique individual identifications, as each individual organism will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individual organisms and 40 from tissue. The nucleic acid sequences of the invention uniquely represent portions of the

human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard
5 against which DNA from an individual organism can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals.

If a panel of reagents from the nucleic acid sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be
10 used to identify tissue from that individual. Using the unique identification database, positive identification of the individual organism, living or dead, can be made from extremely small tissue samples.

DNA-based identification techniques can also be used in forensic biology. Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make
15 such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or skin, or body fluids, *e.g.*, blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.*, another DNA sequence that is unique to a particular individual organism). As mentioned above, actual base sequence information can be used for
25 identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individual organisms using this technique. Examples of polynucleotide reagents include the nucleic acid sequences of the invention or portions thereof, *e.g.*, fragments derived
30 from noncoding regions having a length of at least 20 or 30 bases.

The nucleic acid sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, liver tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin.
35 Panels of such probes can be used to identify tissue by species and/or by organ type.

Antibodies directed against unimpaired or mutant angiotensin gene products or conserved variants or peptide fragments thereof, which are discussed, above, may also be used as diagnostics and prognostics for an angiogenesis-related disorder, *e.g.*, cancer, as described herein. Such methods may be used to detect abnormalities in the level of
40 angiotensin gene product synthesis or expression, or abnormalities in the structure, temporal

expression, and/or physical location of angiostatin gene product. The antibodies and immunoassay methods described below have, for example, important *in vitro* applications in purifying angiostatin gene products and in assessing the efficacy of treatments for angiogenesis-related disorders, e.g., cancer. Antibodies, or fragments of antibodies, such as those described below, may be used to screen potentially therapeutic compounds *in vitro* to determine their effects on angiostatin gene expression and angiostatin peptide production. The compounds that have beneficial effects on an angiogenesis-related disorder, e.g., cancer, can be identified, and a therapeutically effective dose determined.

In vitro immunoassays may also be used, for example, to assess the efficacy of cell-based gene therapy for an angiogenesis-related disorder, e.g., cancer. Antibodies directed against angiostatin peptides may be used *in vitro* to determine, for example, the level of angiostatin gene expression achieved in cells genetically engineered to produce angiostatin peptides. In the case of intracellular angiostatin gene products, such an assessment is done, preferably, using cell lysates or extracts. Such analysis allows for a determination of the number of transformed cells necessary to achieve therapeutic efficacy *in vivo*, as well as optimization of the gene replacement protocol.

The tissue or cell type to be analyzed will generally include those that are known, or suspected, to express the angiostatin gene. The protein isolation methods employed herein may, for example, be such as those described in Harlow and Lane (1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The isolated cells can be derived from cell culture or from a subject. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based gene therapy technique or, alternatively, to test the effect of compounds on the expression of the angiostatin gene.

Preferred diagnostic methods for the detection of angiostatin gene products or conserved variants or peptide fragments thereof, may involve, for example, immunoassays wherein the angiostatin gene products or conserved variants or peptide fragments are detected by their interaction with an anti-angiostatin gene product-specific antibody.

For example, antibodies, or fragments of antibodies, such as those described, above, useful in the present invention may be used to quantitatively or qualitatively detect the presence of angiostatin gene products or conserved variants or peptide fragments thereof. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below, this section) coupled with light microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred for angiostatin gene products that are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of angiostatin gene products or conserved variants or peptide fragments thereof. *In situ* detection may be accomplished by removing a histological specimen from a subject, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is

preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the angiotatin gene product, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Immunoassays for angiotatin gene products or conserved variants or peptide fragments thereof will typically comprise incubating a sample, such as a biological fluid, a tissue extract, freshly harvested cells, or lysates of cells, that have been incubated in cell culture, in the presence of a detectably labeled antibody capable of identifying angiotatin gene products or conserved variants or peptide fragments thereof, and detecting the bound antibody by any of a number of techniques well known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support that is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled angiotatin gene specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-angiotatin gene product antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the angiotatin gene peptide-specific antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, A., *"The Enzyme Linked Immunosorbent Assay (ELISA)"*, 1978, Diagnostic Horizons 2, 1-7, Microbiological Associates Quarterly Publication, Walkersville, MD); Voller, A. et al., 1978, *J. Clin. Pathol.* 31, 507-520; Butler, J.E., 1981, *Meth. Enzymol.* 73, 482-523;

Maggio, E. (ed.), 1980, *Enzyme Immunoassay*, CRC Press, Boca Raton, FL; Ishikawa, E. *et al.*, (eds.), 1981, *Enzyme Immunoassay*, Kigaku Shoin, Tokyo). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety that can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes that can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, α -glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β -galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods that employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection also may be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect angiostatin gene peptides through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, therromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The following assays are designed to identify compounds that bind to an angiostatin gene product, proteins, *e.g.*, intracellular proteins or portions of proteins that interact with an angiostatin gene product, compounds that interfere with the interaction of an angiostatin gene product with intracellular proteins and compounds that modulate the activity of an angiostatin gene (*i.e.*, modulate the level of angiostatin gene expression and/or modulate the level of angiostatin gene product activity). Assays may additionally be utilized that identify compounds that bind to angiostatin gene regulatory sequences (*e.g.*, promoter sequences; see *e.g.*, Platt, 1994, *J. Biol. Chem.* 269, 28558-28562), and that may modulate the level of angiostatin gene expression. Compounds may include, but are not limited to, small organic molecules, such as ones that are able to cross the blood-brain barrier, gain entry into an appropriate cell and affect expression of the angiostatin gene or some other gene involved in an angiostatin regulatory pathway, or intracellular proteins.

Methods for the identification of such intracellular proteins are described, below. Such intracellular proteins may be involved in the control and/or regulation of mood. Further, among these compounds are compounds that affect the level of angiostatin gene expression and/or angiostatin gene product activity and that can be used in the therapeutic treatment of angiostatin disorders, *e.g.*, cancer, as described, below.

Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, and members of random peptide libraries; (see, *e.g.*, Lam, *et al.*, 1991, *Nature* 354, 82-84; Houghten, *et al.*, 1991, *Nature* 354, 84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, *et al.*, 1993, *Cell* 72, 767-778), antibodies (including, but not limited to, polyclonal, monoclonal, human, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')₂ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Such compounds may also include drugs or members of classes or families of drugs, known to ameliorate or exacerbate the symptoms of an angiogenesis-related disorder. Such compounds include, but are not limited to, angiogenesis inhibitors: metalloproteinase inhibitors, FGF and VEGF receptor inhibitors, COX-2 inhibitors, INF, IL-12, Taxol, vinblastine, thalidomide, etc. Preferably such compounds are utilized in a manner (*e.g.*, different dosage, mode of administration, and/or co-administration with one or more additional compounds) that differs from the manner in which such compounds have been administered previously.

Compounds identified via assays such as those described herein may be useful, for example, in elaborating the biological function of the angiostatin gene product, and for ameliorating angiogenesis-related disorders, *e.g.*, cancer. For example, compounds identified via such techniques can provide lead compounds to be tested for an ability to modulate an angiostatin-mediated process and/or to ameliorate symptoms of a angiogenesis-related

disorder. Assays for testing the effectiveness of compounds, identified by, for example, techniques such as those described above, are discussed, below.

In vitro systems may be designed to identify compounds that bind angiostatin gene products of the invention, such as, for example, angiostatin polypeptides. Compounds identified may be useful, for example, in modulating the activity of unimpaired and/or mutant angiostatin gene products, may be useful in elucidating the biological function of the angiostatin gene product, may be utilized in screens for identifying compounds that disrupt normal angiostatin gene product interactions, or may in themselves disrupt such interactions, and can provide lead compounds to be further tested for an ability to modulate an angiostatin-mediated process and/or to ameliorate symptoms of an angiogenesis-related disorder.

The principle of the assays used to identify compounds that bind to angiostatin gene products involves preparing a reaction mixture of the angiostatin gene product and the test compound under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways. For example, one method to conduct such an assay would involve anchoring an angiostatin gene product or the test substance onto a solid phase and detecting angiostatin gene product/test compound complexes anchored on the solid phase at the end of the reaction. In one embodiment of such a method, the angiostatin gene product may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtiter plates may conveniently be utilized as the solid phase. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished by simply coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody, specific for the protein to be immobilized may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the non-immobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the previously non-immobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for angiostatin gene product or the test compound to anchor any complexes

formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Any method suitable for detecting protein-protein interactions may be employed for identifying angiotensin protein-protein interactions.

5 Among the traditional methods that may be employed are co-immunoprecipitation, cross-linking and co-purification through gradients or chromatographic columns. Utilizing procedures such as these allows for the identification of proteins, including intracellular proteins, that interact with angiotensin gene products. Once isolated, such a protein can be identified and can be used in conjunction with standard techniques, to identify proteins it
10 interacts with. For example, at least a portion of the amino acid sequence of a protein that interacts with the angiotensin gene product can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g., Creighton, 1983, *"Proteins: Structures and Molecular Principles,"* W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of
15 oligonucleotide mixtures that can be used to screen for gene sequences encoding such proteins. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the screening are well known. (See, e.g., Ausubel, *supra*, and 1990, *"PCR Protocols: A Guide to Methods and Applications,"* Innis, et al., eds. Academic Press, Inc., New York).

20 Additionally, methods may be employed that result in the simultaneous identification of genes that encode a protein which interacts with an angiotensin protein. These methods include, for example, probing expression libraries with labeled angiotensin protein, using angiotensin protein in a manner similar to the well known technique of antibody probing of λ gt11 libraries.

25 One method that detects protein interactions *in vivo*, the two-hybrid system, is described in detail for illustration only and not by way of limitation. One version of this system has been described (Chien, et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

30 Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to the angiotensin gene product and the other consists of the transcription activator protein's activation domain fused to an unknown protein that is encoded by a cDNA that has been recombined into this plasmid as part of a cDNA library. The DNA-binding domain fusion plasmid and the cDNA library are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (e.g., HBS or *lacZ*) whose regulatory region contains
35 the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the

functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, angiotensin gene products may be used as the bait gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a bait angiotensin gene product fused to the DNA-binding domain are co-transformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, a bait angiotensin gene sequence, such as the open reading frame of the angiotensin gene, can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A cDNA library of the cell line from which proteins that interact with the bait angiotensin gene product can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait angiotensin gene-GAL4 fusion plasmid into a yeast strain that contains a lacZ gene driven by a promoter that contains GAL4 activation sequence. A cDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait angiotensin gene product will reconstitute an active GAL4 protein and thereby drive expression of the HIS3 gene. Colonies that express HIS3 can be detected by their growth on petri dishes containing semi-solid agar based media lacking histidine. The cDNA can then be purified from these strains, and used to produce and isolate the bait angiotensin gene-interacting protein using techniques routinely practiced in the art.

Angiotensin gene products of the invention may, *in vivo*, interact with one or more macromolecules, including cellular or extracellular macromolecules, such as proteins. Such macromolecules may include, but are not limited to, other proteins, such as cellular receptors, or nucleic acid molecules and those proteins identified via methods such as those described, above. For example, the angiotensin gene product may interact with a receptor as a peptide hormone or neuropeptide. For purposes of this discussion, the macromolecules are referred to herein as "binding partners". Compounds that disrupt angiotensin binding in this way may be useful in regulating the activity of the angiotensin gene product, especially mutant angiotensin gene products. For example, such compounds may interfere with the interaction of the angiotensin gene product, with its receptor. Such compounds may include, but are not limited to, molecules such as peptides, and the like, as described, for example, above, which would be capable of gaining access to an angiotensin gene product.

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the angiotensin gene product and its binding partner or partners

involves preparing a reaction mixture containing the angiotensin gene product, and the binding partner under conditions and for a time sufficient to allow the two to interact and bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture, or may be added at a time subsequent to the addition of the angiotensin gene product and its binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the angiotensin gene protein and the binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the angiotensin gene protein and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal angiotensin gene protein may also be compared to complex formation within reaction mixtures containing the test compound and a mutant angiotensin gene protein. This comparison may be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal angiotensin gene proteins.

The assay for compounds that interfere with the interaction of the angiotensin gene products and binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the angiotensin gene product or the binding partner onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the angiotensin gene products and the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the angiotensin gene protein and interactive intracellular binding partner. Alternatively, test compounds that disrupt preformed complexes, e.g., compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are described briefly below.

In a heterogeneous assay system, either the angiotensin gene product or the interactive binding partner, is anchored onto a solid surface, while the non-anchored species is labeled, either directly or indirectly. In practice, microtiter plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the angiotensin gene product or binding partner and drying. Alternatively, an immobilized antibody specific for the species to be anchored may be used to anchor the species to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction components, test compounds that inhibit complex formation or that disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds that inhibit complex or that disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the angiostatin gene protein and the interactive binding partner is prepared in which either the angiostatin gene product or its binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, *e.g.*, U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt a angiostatin gene protein/binding partner interaction can be identified.

In a particular embodiment, the angiostatin gene product can be prepared for immobilization using recombinant DNA techniques described above. For example, the angiostatin coding region can be fused to a glutathione-S-transferase (GST) gene using a fusion vector, such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive binding partner can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art and described above. This antibody can be labeled with the radioactive isotope ¹²⁵I, for example, by methods routinely practiced in the art. In a heterogeneous assay, *e.g.*, the GST-angiostatin fusion protein can be anchored to glutathione-agarose beads. The interactive binding partner can then be added in the presence or absence of the test compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the

complexed components. The interaction between the angiotensinogen protein and the interactive binding partner can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured radioactivity.

5 Alternatively, the GST-angiotensinogen gene fusion protein and the interactive binding partner can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be added either during or after the species are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the angiotensinogen gene product/binding partner
10 interaction can be detected by adding the labeled antibody and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the angiotensinogen protein and/or the interactive or binding partner (in cases where the binding partner is a protein), in
15 place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the binding sites. These methods include, but are not limited to, mutagenesis of the gene encoding one of the proteins and screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the gene encoding the second species in the complex can then be selected. Sequence analysis of the
20 genes encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this section, above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain
25 associated with the solid material, which can be isolated and identified by amino acid sequencing. Also, once the gene coding for the segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

For example, and not by way of limitation, an angiotensinogen gene product can be
30 anchored to a solid material as described, above, in this section by making a GST-angiotensinogen fusion protein and allowing it to bind to glutathione agarose beads. The interactive binding partner obtained can be labeled with a radioactive isotope, such as ^{35}S , and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-angiotensinogen fusion protein and allowed to bind. After washing away unbound peptides,
35 labeled bound material, representing the binding partner binding domain, can be eluted, purified, and analyzed for amino acid sequence by well known methods. Peptides so identified can be produced synthetically or fused to appropriate facilitative proteins using recombinant DNA technology.

Compounds including, but not limited to, binding compounds identified via assay
40 techniques such as those described, above, can be tested for the ability to ameliorate

symptoms of an angiogenesis-related disorder, *e.g.*, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; wound granulation; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; diabetic neovascularization; macular degeneration; fractures; vasculogenesis; hematopoiesis; ovulation; menstruation; placentation; intestinal adhesions; atherosclerosis; scleroderma; hypertrophic scars, *i.e.*, keloids; cat scratch disease (Rochele minalia quintosa); and ulcers (*Helicobacter pylori*). It should be noted that the assays described herein can identify compounds that affect angiostatin gene activity by either affecting angiostatin gene expression or by affecting the level of angiostatin gene product activity. For example, compounds may be identified that are involved in another step in the pathway in which the angiostatin gene and/or angiostatin gene product is involved and, by affecting this same pathway may modulate the effect of angiostatin on the development of an angiogenesis-related disorder such as cancer. Such compounds can be used as part of a therapeutic method for the treatment of the disorder.

Described below are cell-based and animal model-based assays for the identification of compounds exhibiting such an ability to ameliorate symptoms of an angiogenesis-related disorder, *e.g.*, cancer.

First, cell-based systems can be used to identify compounds that may act to ameliorate symptoms of an angiogenesis-related disorder, *e.g.*, cancer. Such cell systems can include, for example, recombinant or non-recombinant cell, such as cell lines, that express the angiostatin gene.

In utilizing such cell systems, cells that express angiostatin may be exposed to a compound suspected of exhibiting an ability to ameliorate symptoms of an angiogenesis-related disorder, *e.g.*, cancer, at a sufficient concentration and for a sufficient time to elicit such an amelioration of such symptoms in the exposed cells. After exposure, the cells can be assayed to measure alterations in the expression of the angiostatin gene, *e.g.*, by assaying cell lysates for angiostatin mRNA transcripts (*e.g.*, by Northern analysis) or for angiostatin gene products expressed by the cell; compounds that modulate expression of the angiostatin gene are good candidates as therapeutics. Alternatively, the cells are examined to determine whether one or more cellular phenotypes associated with an angiogenesis-related disorder, *e.g.*, cancer, has been altered to resemble a more normal or unimpaired, unaffected phenotype, or a phenotype more likely to produce a lower incidence or severity of disorder symptoms.

In addition, animal-based systems or models for an angiogenesis-related disorder, *e.g.*, cancer, may be used to identify compounds capable of ameliorating symptoms of the

disorder. Such animal-based systems or models may include, for example, transgenic mice, *e.g.*, mice that have been genetically engineered to express exogenous or endogenous angiostatin sequences or, alternatively, to no longer express endogenous angiostatin gene sequences (*i.e.*, "knock-out" mice). Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies and interventions that may be effective in treating such disorders. For example, animal models may be exposed to a compound suspected of exhibiting an ability to ameliorate symptoms, at a sufficient concentration and for a sufficient time to elicit such an amelioration of symptoms of an angiogenesis-related disorder, *e.g.*, cancer, in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of such symptoms.

With regard to intervention, any treatments that reverse any aspect of symptoms of an angiogenesis-related disorder, *e.g.*, cancer, should be considered as candidates for human therapeutic intervention in such a disorder. Dosages of test agents may be determined by deriving dose-response curves, as discussed in, below.

A variety of methods can be employed for the diagnostic and prognostic evaluation of angiogenesis-related disorders, such as cancer, and for the identification of subjects having a predisposition to such disorders.

Such methods may, for example, utilize reagents such as the angiostatin gene nucleotide sequences described above, and antibodies directed against angiostatin gene products, including peptide fragments thereof, as described, above. Specifically, such reagents may be used, for example, for:

- (1) the detection of the presence of angiostatin gene mutations, or the detection of either over- or under-expression of angiostatin gene mRNA relative to the state of an angiogenesis-related disorder, such as cancer;
- (2) the detection of either an over- or an under-abundance of angiostatin gene product relative to the unaffected state; and
- (3) the detection of an aberrant level of angiostatin gene product activity relative to the unaffected state.

Angiostatin gene nucleotide sequences can, for example, be used to diagnose an angiogenesis-related disorder using, for example, the techniques for angiostatin mutation detection described above.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific angiostatin gene nucleic acid or anti-angiostatin gene antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings, to diagnose subjects exhibiting abnormalities of an angiogenesis-related disorder, *e.g.*, cancer.

For the detection of angiostatin mutations, any nucleated cell can be used as a starting source for genomic nucleic acid. For the detection of angiostatin gene expression or angiostatin gene products, any cell type or tissue in which the angiostatin gene is expressed may be utilized.

Nucleic acid-based detection techniques are described, above. Peptide detection techniques are described, above.

The methods described herein can furthermore be utilized as diagnostic or prognostic assays to identify subjects having or at risk of developing a disease or disorder associated with aberrant expression or activity of a polypeptide of the invention. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with aberrant expression or activity of a polypeptide of the invention. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing such a disease or disorder. Thus, the present invention provides a method in which a test sample is obtained from a subject and a polypeptide or nucleic acid (e.g., mRNA, genomic DNA) of the invention is detected, wherein the presence of the polypeptide or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant expression or activity of the polypeptide. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (e.g., serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant expression or activity of a polypeptide of the invention. For example, such methods can be used to determine whether a subject can be effectively treated with a specific agent or class of agents (e.g., agents of a type which decrease activity of the polypeptide). Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant expression or activity of a polypeptide of the invention in which a test sample is obtained and the polypeptide or nucleic acid encoding the polypeptide is detected (e.g., wherein the presence of the polypeptide or nucleic acid is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant expression or activity of the polypeptide).

The methods of the invention also can be used to detect genetic lesions or mutations in a gene of the invention, thereby determining if a subject with the lesioned gene is at risk for a disorder characterized aberrant expression or activity of a polypeptide of the invention. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion or mutation characterized by at least one of an alteration affecting the integrity of a gene encoding the polypeptide of the invention, or the mis-expression of the gene encoding the polypeptide of the invention. For example, such genetic lesions or mutations can be detected by ascertaining the existence of at least one of: 1) a deletion of one or more nucleotides from the gene; 2) an addition of one or more nucleotides to the gene; 3) a substitution of one or more nucleotides of the gene; 4) a chromosomal rearrangement of the gene; 5) an alteration in the level of a messenger RNA transcript of the gene; 6) an aberrant modification of the gene, such as of the methylation pattern of the

genomic DNA; 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of the gene; 8) a non-wild type level of a the protein encoded by the gene; 9) an allelic loss of the gene; and 10) an inappropriate post-translational modification of the protein encoded by the gene. As described herein, there are a large number of assay techniques known in the art which can be used for detecting lesions in a gene.

In certain embodiments, detection of the lesion involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) *Science* 241:1077-1080; and Nakazawa et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in a gene (see, e.g., Abravaya et al. (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to the selected gene under conditions such that hybridization and amplification of the gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) *Bio/Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a selected gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, e.g., U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotide probes (Cronin et al., 1996, *Human Mutation* 7:244-255; Kozal et al., 1996, *Nature Medicine* 2:753-759). For example, genetic mutations can be identified in two-dimensional arrays containing light-generated DNA probes as described in Cronin et al., *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches

of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the selected gene and detect mutations by comparing the sequence of the sample nucleic acids with the corresponding wild-type (control) sequence. (Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert, 1977, *Proc. Natl. Acad. Sci. USA* 74:560 or Sanger, 1977, *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (1995, *Bio/Techniques* 19:448), including sequencing by mass spectrometry (*see, e.g.*, PCT Publication No. WO 94/16101; Cohen et al., 1996, *Adv. Chromatogr.* 36:127-162; and Griffin et al., 1993, *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in a selected gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al., 1985, *Science* 230:1242). In general, the technique of mismatch cleavage entails providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. RNA/DNA duplexes can be treated with RNase to digest mismatched regions, and DNA/DNA hybrids can be treated with S1 nuclease to digest mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. (*See, e.g.*, Cotton et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:4397; Saleeba et al., 1992, *Methods Enzymol.* 217:286-295.) In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair enzymes") in defined systems for detecting and mapping point mutations in cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al., 1994, *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a selected sequence, *e.g.*, a wild-type sequence, is hybridized

to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. (See, e.g., U.S. Patent No. 5,459,039.)

5 In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in genes. For example, SSCP may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:2766; see also Cotton, 1993, *Mutat. Res.* 285:125-144; Hayashi, 1992, *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic
10 acids varies according to sequence, and the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to
15 separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al., 1991, *Trends Genet.* 7:5).

In yet another embodiment, the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al., 1985, *Nature* 313:495). When DGGE is used as
20 the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner, 1987, *Biophys. Chem.* 265:12753).

25 Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al., 1986, *Nature* 324:163; Saiki et al.,
30 1989, *Proc. Natl. Acad. Sci. USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR
35 amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al., 1989, *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent or reduce polymerase extension (Prossner,
40 1993, *Tibtech* 11:238). In addition, it may be desirable to introduce a novel restriction site in

the region of the mutation to create cleavage-based detection (Gasparini et al., 1992, *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany, 1991, *Proc. Natl. Acad. Sci. USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification. The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose subjects exhibiting symptoms or family history of a disease or illness involving a gene encoding a polypeptide of the invention. Furthermore, any cell type or tissue, preferably peripheral blood leukocytes, in which the polypeptide of the invention is expressed may be utilized in the prognostic assays described herein.

The invention further provides kits that facilitate the use and/or detection of angiostatin genes and gene products described herein. The kits described herein may be conveniently used, e.g., in clinical settings to diagnose subjects exhibiting symptoms or family history of a disease or illness involving a gene encoding a polypeptide of the invention. Furthermore, any cell type or tissue in which the polypeptide of the invention is expressed may be utilized in the prognostic assays described herein. In one embodiment, a diagnostic test kit for identifying cells or tissues which mis-express angiostatin genes or gene products is provided. In this embodiment, a diagnostic kit is provided, with one or more containers comprising an oligonucleotide, e.g., a detectably labeled oligonucleotide, which hybridizes to a nucleic acid sequence encoding a polypeptide of the invention. In another embodiment, a kit is provided, with one or more containers comprising a pair of primers useful for amplifying a nucleic acid molecule encoding a polypeptide of the invention. In various other embodiments, the kit can also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kit also can comprise components necessary for detecting the detectable agent (e.g., an enzyme or a substrate). The kit also can contain a control sample or a series of control samples which can be assayed and compared to the test sample. Each component of the kit is usually enclosed within an individual container and all of the various containers are within a single package along with instructions for observing whether the tested subject is suffering from, or is at risk of developing, a disorder associated with aberrant expression of the polypeptide. Such a kit can be used, for example, to measure the levels of a nucleic acid molecule encoding the protein in a sample of cells from a subject, e.g., detecting mRNA levels or determining whether a gene encoding the protein has been mutated or deleted.

In another embodiment, the invention provides kits for detecting the presence of a polypeptide or nucleic acid of the invention in a biological sample (a test sample). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant expression of a polypeptide of the invention as discussed, for example, in sections above relating to uses of the sequences of the invention. In this embodiment, a kit is provided, with one or more containers comprising: (1) a first antibody

(*e.g.*, attached to a solid support) which binds to a polypeptide of the invention; and, optionally, (2) a second, different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable agent. Such kits can be used to determine if a subject is suffering from, or is at increased risk of, an angiogenesis-related disorder, such as cancer.

5 Described below are methods and compositions whereby an angiostatin-mediated process can be modulated and/or whereby an angiogenesis-related disorder, *e.g.*, cancer, may be treated.

For example, such methods can comprise administering compounds which modulate the expression of an angiostatin gene and/or the synthesis or activity of an angiostatin gene product so that the process is modulated or a symptom of the disorder is ameliorated.

10 Alternatively, in those instances whereby the angiogenesis-related disorder, *e.g.*, cancer, results from angiostatin gene mutations that lower or abolish angiostatin activity, respectively, such methods can comprise supplying a mammal with a nucleic acid molecule encoding an unimpaired angiostatin gene product such that an unimpaired angiostatin gene product is expressed and symptoms of the disorder are ameliorated.

15 In another embodiment of methods for the treatment of mammalian angiogenesis-related disorder, *e.g.*, cancer, resulting from angiostatin gene mutations, such methods can comprise supplying a mammal with a cell comprising a nucleic acid molecule that encodes an unimpaired angiostatin gene product such that the cell expresses the unimpaired angiostatin gene product and symptoms of the disorder are ameliorated.

20 In cases in which a loss of normal angiostatin gene product function results in the development of an angiogenesis-related disorder phenotype, *e.g.*, cancer, an increase in angiostatin gene product activity would facilitate progress towards an asymptomatic state in individual organisms exhibiting a deficient level of angiostatin gene expression and/or angiostatin gene product activity. Methods for enhancing the expression or synthesis of angiostatin can include, for example, methods such as those described below.

25 Alternatively, symptoms of angiogenesis-related disorder phenotype, *e.g.*, cancer, may be ameliorated by administering a compound that decreases the level of angiostatin gene expression and/or angiostatin gene product activity. Methods for inhibiting or reducing the level of angiostatin synthesis or expression can include, for example, methods such as those described below.

30 In one embodiment of treatment methods, the compounds administered do not comprise compounds, in particular drugs, reported to ameliorate or exacerbate the symptoms of an angiogenesis-related disorder. If such treatment methods do comprise such compounds, preferably such compounds are utilized in a manner (*e.g.*, different dosage, mode of administration, and/or co-administration with one or more additional compounds) that differs from the manner in which such compounds have been administered previously.

35 In another embodiment, symptoms of a disorder described herein, *e.g.*, cancer, may be ameliorated by angiostatin protein therapy methods, *e.g.*, decreasing or increasing the level and/or activity of angiostatin using the angiostatin protein, fusion protein, and peptide

sequences described above, or by the administration of proteins or protein fragments (*e.g.*, peptides) which interact with an angiotatin gene or gene product and thereby inhibit or potentiate its activity.

5 Such protein therapy may include, for example, the administration of a functional angiotatin protein or fragments of an angiotatin protein (*e.g.*, peptides) which represent functional angiotatin domains.

10 In one embodiment, angiotatin fragments or peptides representing a functional angiotatin binding domain are administered to an individual organism such that the peptides bind to an angiotatin binding protein, *e.g.*, an angiotatin receptor. Such fragments or peptides may serve to inhibit angiotatin activity in an individual organism by competing with, and thereby inhibiting, binding of angiotatin to the binding protein, thereby ameliorating symptoms of a disorder described herein. Alternatively, such fragments or peptides may enhance angiotatin activity in an individual organism by mimicking the function of angiotatin *in vivo*, thereby ameliorating the symptoms of a disorder described herein.

15 The proteins and peptides which may be used in the methods of the invention include synthetic (*e.g.*, recombinant or chemically synthesized) proteins and peptides, as well as naturally occurring proteins and peptides. The proteins and peptides may have both naturally occurring and non-naturally occurring amino acid residues (*e.g.*, D-amino acid residues) and/or one or more non-peptide bonds (*e.g.*, imino, ester, hydrazide, semicarbazide, and azo bonds). The proteins or peptides may also contain additional chemical groups (*i.e.*, functional groups) present at the amino and/or carboxy termini, such that, for example, the stability, bioavailability, and/or inhibitory activity of the peptide is enhanced. Exemplary functional groups include hydrophobic groups (*e.g.* carbobenzoxy, dansyl, and t-butyloxycarbonyl, groups), an acetyl group, a 9-fluorenylmethoxy-carbonyl group, and macromolecular carrier groups (*e.g.*, lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates) including peptide groups.

25 In another embodiment, symptoms of certain angiogenesis-related disorders, such as cancer, may be ameliorated by decreasing the level of angiotatin gene expression and/or angiotatin gene product activity by using angiotatin gene sequences in conjunction with well-known antisense, gene "knock-out," ribozyme and/or triple helix methods to decrease the level of angiotatin gene expression. Among the compounds that may exhibit the ability to modulate the activity, expression or synthesis of the angiotatin gene, including the ability to ameliorate the symptoms of an angiogenesis-related disorder, *e.g.*, cancer, are antisense, ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit 35 either unimpaired, or if appropriate, mutant target gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides that are complementary to a target gene mRNA. The

antisense oligonucleotides will bind to the complementary target gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required.

5 A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled
10 in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In one embodiment, oligonucleotides complementary to non-coding regions of the angiotatin gene could be used in an antisense approach to inhibit translation of endogenous angiotatin mRNA. Antisense nucleic acids should be at least six nucleotides in length, and
15 are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene
20 expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is
25 preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or
30 modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger, *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86, 6553-6556; Lemaitre, *et al.*, 1987, *Proc. Natl. Acad. Sci.* 84, 648-652; PCT
35 Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, e.g., Krol *et al.*, 1988, *BioTechniques* 6, 958-976) or intercalating agents (see, e.g., Zon, 1988, *Pharm. Res.* 5, 539-549). To this end, the oligonucleotide may

be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxymethylaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier, *et al.*, 1987, *Nucl. Acids Res.* 15, 6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue, *et al.*, 1987, *Nucl. Acids Res.* 15, 6131-6148), or a chimeric RNA-DNA analogue (Inoue, *et al.*, 1987, *FEBS Lett.* 215, 327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Blosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein, *et al.* (1988, *Nucl. Acids Res.* 16, 3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin, *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85, 7448-7451), etc.

While antisense nucleotides complementary to the target gene coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

Antisense molecules should be delivered to cells that express the target gene *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells;

e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

5 However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the subject will result in the transcription of sufficient amounts of single stranded RNAs
10 that will form complementary base pairs with the endogenous target gene transcripts and thereby prevent translation of the target gene mRNA. For example, a vector can be introduced e.g., such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed
15 by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region
20 (Benoist and Chambon, 1981, *Nature* 290, 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, *Cell* 22, 787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78, 1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, 1982, *Nature* 296, 39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the
25 recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used that selectively infect the desired tissue, in which case administration may be accomplished by another route (e.g., systemically).

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of
30 target gene product. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver, *et al.*, 1990, *Science* 247, 1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, *Current Biology* 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to
35 complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, e.g., U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially FIG. 4, page 833) and in Haseloff and Gerlach, 1988, *Nature*, 334, 585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target gene mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, *Science*, 224, 574-578; Zaug and Cech, 1986, *Science*, 231, 470-475; Zaug, *et al.*, 1986, *Nature*, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, *Cell*, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies, *et al.*, 1985, *Nature* 317, 230-234; Thomas and Capecchi, 1987, *Cell* 51, 503-512; Thompson, *et al.*, 1989, *Cell* 5, 313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate

animal offspring with an inactive target gene (e.g., see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

5 Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (i.e., the target gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene in target cells in the body. (See generally, Helene, 1991, *Anticancer Drug Des.*, 6(6), 569-584; Helene, *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 660, 27-36; and Maher, 1992, *Bioassays* 14(12), 807-815).

10 Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be
15 pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for
20 example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

25 Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

30 In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles that the possibility may arise wherein the concentration of normal target gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels
35 of target gene activity are maintained, therefore, nucleic acid molecules that encode and express target gene polypeptides exhibiting normal target gene activity may, be introduced into cells via gene therapy methods such as those described, below, that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the target gene encodes an extracellular protein, it

may be preferable to co-administer normal target gene protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyri-
bonucleotides and oligoribonucleotides well known in the art such as for example solid phase
phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in*
vitro and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such
DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable
RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively,
antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly,
depending on the promoter used, can be introduced stably into cell lines.

With respect to an increase in the level of normal angiostatin gene expression and/or
angiostatin gene product activity, angiostatin gene nucleic acid sequences, described above,
for example, can be utilized for the treatment of an angiogenesis-related disorder, *e.g.*,
cancer. Such treatment can be administered, for example, in the form of gene replacement
therapy. Specifically, one or more copies of a normal angiostatin gene or a portion of the
angiostatin gene that directs the production of a angiostatin gene product exhibiting normal
angiostatin gene function, may be inserted into the appropriate cells within a subject, using
vectors that include, but are not limited to, adenovirus, adeno-associated virus, herpesvirus
and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as
liposomes.

Because angiostatin genes can be expressed in the brain, such gene replacement
therapy techniques should be capable delivering angiostatin gene sequences to these cell
types within subjects. Thus, in one embodiment, techniques that are well known to those of
skill in the art (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988) can be
used to enable angiostatin gene sequences to cross the blood-brain barrier readily and to
deliver the sequences to cells in the brain. With respect to delivery that is capable of crossing
the blood-brain barrier, viral vectors such as, for example, those described above, are
preferable.

In another embodiment, techniques for delivery involve direct administration of such
angiostatin gene sequences to the site of the cells in which the angiostatin gene sequences
are to be expressed.

Additional methods that may be utilized to increase the overall level of angiostatin
gene expression and/or angiostatin gene product activity include the introduction of
appropriate angiostatin-expressing cells, preferably autologous cells, into a subject at
positions and in numbers that are sufficient to ameliorate the symptoms of an angiogenesis-
related disorder, *e.g.*, cancer. Such cells may be either recombinant or non-recombinant.

Among the cells that can be administered to increase the overall level of angiostatin gene expression in a subject are normal cells, preferably liver cells, that express the angiostatin gene.

Alternatively, cells, preferably autologous cells, can be engineered to express angiostatin gene sequences, and may then be introduced into a subject in positions appropriate for the amelioration of the symptoms of an angiogenesis-related disorder, e.g., cancer. Alternately, cells that express an unimpaired angiostatin gene and that are from an MHC matched individual organism can be utilized, and may include, for example, liver cells. The expression of the angiostatin gene sequences is controlled by the appropriate gene regulatory sequences to allow such expression in the necessary cell types. Such gene regulatory sequences are well known to the skilled artisan. Such cell-based gene therapy techniques are well known to those skilled in the art, see, e.g., Anderson, U.S. Patent No. 5,399,349.

When the cells to be administered are non-autologous cells, they can be administered using well known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Additionally, compounds, such as those identified via techniques such as those described above, that are capable of modulating angiostatin gene product activity can be administered using standard techniques that are well known to those of skill in the art. In instances in which the compounds to be administered are to involve an interaction with brain cells, the administration techniques should include well known ones that allow for a crossing of the blood-brain barrier.

Agents, or modulators, which have a stimulatory or inhibitory effect on activity or expression of a polypeptide of the invention as identified by a screening assay described herein can be administered to individual organisms to treat (prophylactically or therapeutically) disorders associated with aberrant activity of the polypeptide. In conjunction with such treatment, the pharmacogenomics (i.e., the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual organism may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, the pharmacogenomics of the individual organism permits the selection of effective agents (e.g., drugs) for prophylactic or therapeutic treatments based on a consideration of the individual organism's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the activity of a polypeptide of the invention, expression of a nucleic acid of the invention, or mutation content of a gene of the invention in an individual organism can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual organism.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected individual organisms. See, e.g., Linder (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body are referred to as "altered drug action." Genetic conditions transmitted as single factors altering the way the body acts on drugs are referred to as "altered drug metabolism". These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g., N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some subjects do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, a PM will show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification. Thus, the activity of a polypeptide of the invention, expression of a nucleic acid encoding the polypeptide, or mutation content of a gene encoding the polypeptide in an individual organism can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual organism. In addition, pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the identification of an individual organism's drug responsiveness phenotype. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a modulator of activity or expression of the polypeptide, such as a modulator identified by one of the exemplary screening assays described herein.

Monitoring the influence of agents (e.g., drugs, compounds) on the expression or activity of a polypeptide of the invention (e.g., the ability to modulate aberrant cell proliferation chemotaxis, and/or differentiation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent, as determined by a screening assay as described herein, to increase gene expression, protein levels or protein activity, can be monitored in clinical trials of subjects exhibiting decreased gene expression, protein levels, or protein activity. Alternatively, the effectiveness of an agent, as determined by a screening assay, to decrease gene expression, protein levels or protein activity, can be monitored in clinical trials of subjects exhibiting increased gene expression, protein levels, or protein activity. In such clinical trials, expression or activity of a polypeptide of the invention and preferably, that of other polypeptide that have been implicated in for example, a cellular proliferation disorder, can be used as a marker of the immune responsiveness of a particular cell.

For example, and not by way of limitation, genes, including those of the invention, that are modulated in cells by treatment with an agent (e.g., compound, drug or small molecule) which modulates activity or expression of a polypeptide of the invention (e.g., as identified in a screening assay described herein) can be identified. Thus, to study the effect of agents on cellular proliferation disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of a gene of the invention and other genes implicated in the disorder. The levels of gene expression (i.e., a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of a gene of the invention or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during, treatment of the individual organism with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of the polypeptide or nucleic acid of the invention in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of the polypeptide or nucleic acid of the invention in the post-administration samples; (v) comparing the level of the polypeptide or nucleic acid of the invention in the pre-administration sample with the level of the polypeptide or nucleic acid of the invention in the post-administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of the polypeptide to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, decreased administration of the agent

may be desirable to decrease expression or activity of the polypeptide to lower levels than detected, *i.e.*, to decrease the effectiveness of the agent.

The compounds of this invention can be formulated and administered to inhibit a variety of angiogenesis-related disorders by any means that produces contact of the active
5 Ingredient with the agent's site of action in the body of a mammal. They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical
10 practice.

The dosage administered will be a therapeutically effective amount of the compound sufficient to result in amelioration of symptoms of the angiogenesis-related disorder and will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient and its mode and route of administration; age, sex, health
15 and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment, frequency of treatment and the effect desired.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective
20 in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby,
25 reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans and cats. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed
30 and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be
35 used to more accurately determine useful doses in humans and cats. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Specific dosages may also be utilized for antibodies. Typically, the preferred dosage is 0.1 mg/kg to 100 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg), and if the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. If the
40 antibody is partially human or fully human, it generally will have a longer half-life within the

human body than other antibodies. Accordingly, lower dosages of partially human and fully human antibodies is often possible. Additional modifications may be used to further stabilize antibodies. For example, lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described
5 by Cruikshank et al. ((1997) *J. Acquired Immune Deficiency Syndromes and Human Retrovirology* 14:193).

A therapeutically effective amount of protein or polypeptide (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1
10 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight.

Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide or antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject (e.g. a cat) is treated with antibody, protein, or polypeptide in the range of between about 0.1 to 20 mg/kg body weight, one time per week for
15 between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5 or 6 weeks.

The present invention further encompasses agents which modulate expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs,
20 polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds
25 having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

It is understood that appropriate doses of small molecule agents depends upon a number of factors known to those of ordinary skill in the art, e.g., a physician. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of
30 the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention. Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram,
35 about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions.

Solutions for parenteral administration contain preferably a water soluble salt of the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium ethylenediaminetetraacetic acid (EDTA). In addition, parenteral solutions can contain
5 preservatives such as benzalkonium chloride, methyl- or propyl-paraben and chlorobutanol. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field.

The compounds may also be formulated in rectal compositions such as suppositories
10 or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection.
15 Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control release preparations
20 and can include appropriate macromolecules, for example polymers, polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release.

Additionally, the agent can be incorporated into particles of polymeric materials such
25 as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also be used to trap the compound in microcapsules.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may
30 for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Useful pharmaceutical dosage forms, for administration of the compounds of this invention can be illustrated as follows:

Capsules: Capsules are prepared by filling standard two-piece hard gelatin capsules
35 each with the desired amount of powdered active ingredient, 175 milligrams of lactose, 24 milligrams of talc and 6 milligrams magnesium stearate.

Soft Gelatin Capsules: A mixture of active ingredient in soybean oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing the desired amount of the active ingredient. The capsules are then washed and
40 dried.

Tablets: Tablets are prepared by conventional procedures so that the dosage unit is the desired amount of active ingredient. 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 275 milligrams of microcrystalline cellulose, 11 milligrams of cornstarch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or to delay absorption.

Injectable: A parenteral composition suitable for administration by injection is prepared by stirring 1.5% by weight of active ingredients in 10% by volume propylene glycol and water. The solution is made isotonic with sodium chloride and sterilized.

Suspension: An aqueous suspension is prepared for oral administration so that each 5 milliliters contain 100 milligrams of finely divided active ingredient, 200 milligrams of sodium carboxymethyl cellulose, 5 milligrams of sodium benzoate, 1.0 grams of sorbitol solution U.S.P. and 0.025 milliliters of vanillin.

Gene Therapy Administration: Where appropriate, the gene therapy vectors can be formulated into preparations in solid, semisolid, liquid or gaseous forms such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, and aerosols, in the usual ways for their respective route of administration. Means known in the art can be utilized to prevent release and absorption of the composition until it reaches the target organ or to ensure timed-release of the composition. A pharmaceutically acceptable form should be employed which does not ineffectuate the compositions of the present invention. In pharmaceutical dosage forms, the compositions can be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds.

Accordingly, the pharmaceutical composition of the present invention may be delivered via various routes and to various sites in an animal body to achieve a particular effect (see, e.g., Rosenfeld et al. (1991), supra; Rosenfeld et al., Clin. Res., 39(2), 31-41A (1991 a); Jaffe et al., supra; Berkner, supra). One skilled in the art will recognize that although more than one route can be used for administration, a particular route can provide a more immediate and more effective reaction than another route. Local or systemic delivery can be accomplished by administration comprising application or instillation of the formulation into body cavities, inhalation or insufflation of an aerosol, or by parenteral introduction, comprising intramuscular, intravenous, peritoneal, subcutaneous, intradermal, as well as topical administration.

The compositions of the present invention can be provided in unit dosage form wherein each dosage unit, e.g., a teaspoonful, tablet, solution, or suppository, contains a predetermined amount of the composition, alone or in appropriate combination with other active agents. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the compositions of the present invention, alone or in combination with other active agents, calculated in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier, or vehicle, where appropriate.

The specifications for the unit dosage forms of the present invention depend on the particular effect to be achieved and the particular pharmacodynamics associated with the pharmaceutical composition in the particular host.

Accordingly, the present invention also provides a method of transferring a therapeutic
5 gene to a host, which comprises administering the vector of the present invention, preferably as part of a composition, using any of the aforementioned routes of administration or alternative routes known to those skilled in the art and appropriate for a particular application. The "effective amount" of the composition is such as to produce the desired effect in a host which can be monitored using several end-points known to those skilled in the art. Effective
10 gene transfer of a vector to a host cell in accordance with the present invention to a host cell can be monitored in terms of a therapeutic effect (e.g. alleviation of some symptom associated with the particular disease being treated) or, further, by evidence of the transferred gene or expression of the gene within the host (e.g., using the polymerase chain reaction in conjunction with sequencing, Northern or Southern hybridizations, or transcription assays to
15 detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, mRNA or protein half-life studies, or particularized assays to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted in level or function due to such transfer).

These methods described herein are by no means all-inclusive, and further methods
20 to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

Furthermore, the actual dose and schedule can vary depending on whether the compositions are administered in combination with other pharmaceutical compositions, or
25 depending on individual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in in vitro applications depending on the particular cell line utilized (e.g., based on the number of adenoviral receptors present on the cell surface, or the ability of the particular vector employed for gene transfer to replicate in that cell line). Furthermore, the amount of vector to be added per cell will likely vary with the length and stability of the
30 therapeutic gene inserted in the vector, as well as also the nature of the sequence, and is particularly a parameter which needs to be determined empirically, and can be altered due to factors not inherent to the methods of the present invention (for instance, the cost associated with synthesis). One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

35 The following examples are offered by way of example, and are not intended to limit the scope of the invention in any manner.

EXAMPLESIDENTIFICATION AND CLONING OF ANGIOSTATIN GENE

5 In the Example presented in this section, studies are described that identify a novel feline gene, referred to herein as angiostatin, which is involved in angiogenesis-related disorders, e.g., cancer.

MATERIALS AND METHODS

1. Isolation of RNA from cat liver tissue. 50 mg of cat liver tissue was disrupted and homogenized by rotor-stator homogenizer (Kontes, Vineland, NJ) and total RNA was purified using RNeasy Mini Kit following instructions from manufacturer (Qiagen Inc., Santa Clarita, CA). 30 μ g of RNA was isolated and was suspended in H₂O at 0.5 μ g/ μ l.

2. RT-PCR amplification and cloning of a region of feline plasminogen encompassing angiostatin. Angiostatin is an internal fragment of plasminogen (amino acid Val₉₈-Gly₄₅₉ in murine plasminogen). A pair of primers were designed to amplify a region of feline plasminogen cDNA based on consensus sequences from human (accession number X05199), mouse (accession number J04766) and bovine (accession number X79402). The 5' primer: TGCAGGGCATTCCAGTACCACAGTAAAGAGC (SEQ ID NO: 5), corresponding to nucleotide #205-235 of murine plasminogen cDNA, and the 3' primer:

20 ATGCTGTGTCTGTGGGGCTCCTGGGC (SEQ ID NO: 6), corresponding to nucleotide #1543-1568 of murine plasminogen cDNA were used for the amplification reactions. The RT-PCR reaction was performed using Ready-To-Go RT-PCR beads from Amersham Pharmacia Biotech. Inc. 0.5 μ g of cat liver RNA was denatured at 68°C for 2 minutes. The reverse transcription reaction was performed at 42°C for 30 minutes, and the PCR program was: hold at 94°C for 3 minutes, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 1.5 minutes at 68°C and 5 minutes of extension at 68°C. The PCR products were analyzed by electrophoresis.

The PCR products were cloned into Eukaryotic TA cloning vector pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) following manufacturer's instructions and designated as pCR2.1-pro-fe-angiostatin. Three independent clones containing inserts of the expected size (1.3 kb) were sequenced (Advanced Genetic Analysis Center, St. Paul, MN). The sequences were assembled and analyzed using DNASTar (DNASTar Inc. Madison, WI).

3. Subcloning of HA-tagged feline angiostatin. The exact fragment of feline plasminogen corresponding to angiostatin was again cloned into pDisplay vector (Invitrogen, Carlsbad, CA) by PCR amplification from pCR2.1-pro-fe-angiostatin using primers: 5' primer- GTGCCCCGGGATCTATCTTTCAGAGTGCAAGATT (SEQ ID NO: 7), 3' primer- GGAAGTCGACTCACTCTTCTGTCCGGACATTTTTCAGG (SEQ ID NO: 8). To facilitate cloning, two restriction enzyme sites, Xma 1 (5' primer) and Acc 1(3' primer) were incorporated into the primer sequences as shown by underline. The insert was fused in-frame

40 to the signal peptide and HA epitope sequences present in the vector. The vector pDisplay

also contains the platelet derived growth factor receptor (PDGFR) transmembrane domain which can be fused to the C-terminus of cloned protein. However, a stop codon TGA (shown in bold) was added to the 3' primer to terminate translation, therefore the PDGFR transmembrane domain downstream of the insert would not be translated in the final plasmid construct pDisplay-HA-fe-angiotatin.

4. Subcloning of feline angiotatin (without HA tag). Feline angiotatin was cloned into pSecTag2 B vector (Invitrogen, Carlsbad, CA) by PCR amplification of pCR2.I-pro-fe-angiotatin using primers: 5' primer-GACCAAGCTTATCTATCTTTTCAGAGTGCAAGATT (SEQ ID NO: 9), 3' primer-GAACCTCGAGTCACTCTTCTCTGTCCGGACATTTTTCAGG (SEQ ID NO: 10). To facilitate cloning, two restriction enzyme sites, Hind III (5' primer) and Xho I (3' primer) were incorporated into the primer sequences as shown by underline. The insert was fused in-frame to the signal peptide sequences present in the vector. A stop codon TGA (shown in bold) was added to the 3' primer to terminate translation. The final plasmid construct was designated pSecTag2-fe-angiotatin.

5. Cloning of murine angiotatin. In order to compare the antiangiogenesis activity of cloned feline angiotatin to that of its murine counterpart, cDNAs encoding murine angiotatin were RT-PCR amplified from mouse liver RNA and cloned into pDisplay vector. The primers used for amplifying murine angiotatin were: 5'-

GCATAGATCTGTGTATCTGTCAGAATGTAAG (SEQ ID NO: 11), 3'-

CGTAGTCGACCTACCCTCCTGTCTCTGAGCACCGCTTC (SEQ ID NO: 12). The flanking restriction sites Bgl II (5' primer) and Sal I (3' primer) are underlined and the stop codon is shown in bold. The resultant plasmids were designated as pDisplay-HA-mu-angiotatin.

The cDNAs encoding a fragment of feline plasminogen which contains the coding region of angiotatin were amplified by RT-PCR from cat liver mRNA using primers designed from consensus sequences from several species. A 1.5 kb PCR product was amplified from feline plasminogen which was designated pro-angiotatin. The PCR products were cloned into vector pCR 3.1 and sequenced. The nucleotide sequence of pro-angiotatin is shown in Fig. 1 (SEQ ID NO: 1), and the predicted amino acid sequence is shown in Fig. 2 (SEQ ID NO: 2). The region corresponding to angiotatin based on homology is in bold in Fig. 2, and the exact nucleotide sequence and predicated amino acid sequence of feline angiotatin is shown in Fig. 3 (SEQ ID NO: 3) and Fig. 4 (SEQ ID NO: 4) respectively. A comparison of all known amino acid sequences of angiotatin is summarized in Fig. 5. The degree of homology between feline angiotatin and that of human, mouse and bovine is 80%, 78% and 81% respectively. The four kringle domains of plasminogen which are the signature of angiotatin are conserved in all species.

EXPRESSION OF ANGIOSTATIN GENE

In the Example presented in this section, studies are described that identify methods to express and assay the novel feline angiotatin gene.

MATERIALS AND METHODS

- 5 1. Transfection of angiotatin. Human 293 cells grown in 6 well plate were transfected with 2.5 ug of plasmids encoding feline or murine angiotatin using CalPhos Mammalian Transfection Kit (CLONTECH Laboratories, Inc., Palo Alto, CA).
2. Detection of angiotatin by immunoblot analysis. 2 days post-transfection, cells were harvested by lysis in Tris-Glycine SDS Sample Buffer (NOVEX, San Diego, CA). The culture
10 supernatants were harvested by centrifugation at 3000 rpm for 15 minutes. The proteins were separated by 4-12% SDS-PAGE and transferred to PVDF membrane (NOVEX, San Diego, CA). For immunoblot analysis, HA antibody against HA epitope were diluted 1:500 and incubated with the blot for 1.5 hours. After incubating for 30 minutes with alkaline phosphatase conjugated anti-mouse IgG (1:10,000, Boehringer Mannheim, Indianapolis, IN),
15 the bound antibody was detected using phosphatase substrate BCIP/NBT (KPL, Gaithersburg, Maryland).
3. Endothelial cell proliferation assay. The anti-proliferative effect of cloned feline angiotatin was tested using bovine pulmonary artery endothelial cells (C-PAE, ATCC, Manassas, VA). The cells (10^4 cells/well) were plated in 24-well collagen I-coated plates
20 (Collaborative Biomedical Products, Bedford, MA) in OptiMEM (GIBCO BRL, Rockville, MD) with 2% fetal bovine serum (Nova-Tech Inc., Grand Island, NE). After 24 hour incubation, the medium was replaced with conditioned medium from transfected 293 cells supplemented with 1 ng/ml bFGF (Collaborative Biomedical Products, Bedford, MA). The cells were trypsinized 48 hours later and viable cells were counted using trypan blue staining and hemacytometer.
25 The results were analyzed using Prism 2.01 (Graphpad Software Inc., San Diego, CA).

RESULTS

Expression of feline angiotatin. The cDNAs encoding feline angiotatin were subcloned into mammalian expression vector pDisplay. Because angiotatin is a fragment of a secreted protein and normally circulates in the blood, the proteins were fused in-frame at the
30 N-terminus to the murine Ig k-chain leader sequence which directs the protein to the secretory pathway, followed by fusion to hemagglutinin A epitope tag (HA) which allows for detection of expressed protein. Human 293 cells were transfected with plasmids encoding signal sequence and HA-tagged angiotatin from cat and mouse, the cells were harvested 48 hours post transfection for analysis. A 52 kDa protein was detected by HA antibody from both
35 culture supernatants (Fig. 6, lane 3) and cell lysates (Fig. 7, lane 3), indicating that angiotatin was expressed and secreted as expected. It should be noted that the predicted size for HA-tagged angiotatin is 43 kDa, which is smaller than the observed molecular weight of 52 kDa. The increase in molecular weight of exogenously expressed angiotatin has been reported and it could be due to differential glycosylation in different cell types. Nevertheless, such

increase in size does not appear to affect its biological activity (Wu et al., 1997, *Biochem Biophys Res Commun* 236(3):651-4).

Inhibition of endothelial cell proliferation. One unique feature of angiostatin is its ability to specifically inhibit endothelial cell proliferation (O'Reilly et al., 1997, *Cell* 88(2):277-85; O'Reilly et al., 1994, *Cell* 79(2):3 15-28). Bovine pulmonary artery endothelial cells (CPAE cells) (Dhanabal et al., 1999, *J. Biol Chem* 274(17):11721-6) were stimulated with basic fibroblast growth factor (bFGF) in the presence or absence of angiostatin proteins produced from conditioned media from transfected 293 cells. The proliferation rate was normalized against control cells which were treated with bFGF and conditioned media from green fluorescent protein (GFP) transfected cells. The results from four independent experiments were summarized in Fig. 8. CPAE cells proliferated slowly without bFGF activation (52% of that of control cells stimulated with bFGF). The addition of HA-tagged feline angiostatin inhibited the stimulating effect of bFGF with CPAE cells proliferating at 57% of that of the bFGF-stimulated control cells. The inhibitory activity seen with HA-tagged feline angiostatin was comparable to that with the murine protein (59% of the level of controls). The differences in proliferation rates between the positive control and each treated group were statistically significant ($P < 0.0001$). In contrast, treatment with angiostatin did not inhibit proliferation of the epithelial 293 cells, suggesting that the inhibitory effect of angiostatin is specific to endothelial cells. Similar results were obtained using untagged feline angiostatin (Fig. 9). The addition of feline angiostatin specifically inhibited the stimulating effect of bFGF with CPAE cells proliferating at 52% of that of the control. The difference in the proliferation rate between the positive control and the treatment group was statistically significant ($P < 0.0001$).

DISCUSSION

The cloning of feline angiogenesis inhibitor angiostatin is reported here. It shares approximately 78-81% identity at the amino acid level with its human and murine counterparts. To facilitate secretion of cloned proteins, a signal sequence from mouse Ig k-chain was fused to the N-terminus of angiostatin. Immunofluorescent studies and immunoblot assays confirmed that the proteins were localized to the secretory pathway and secreted into conditioned media. Feline angiostatin was also shown to specifically inhibit endothelial cell proliferation at a level comparable to its murine counterparts.

Mouse angiostatin has been shown to inhibit the growth of a wide variety of primary and metastatic tumors. Furthermore, the treatment with angiostatin can be repeated many times without inducing drug resistance or side effects. Since these angiogenesis inhibitors are directed at a novel target (endothelial cells), they can also be conveniently combined with other cancer therapies such as surgery, chemotherapy, radiation therapy and immunotherapy to achieve superior therapeutic effects. These properties have made angiostatin a very attractive candidate for treating feline cancers, where a safe, efficacious, and broad-spectrum therapy is very much in need. However, the successful application of angiostatin as cancer therapy probably will involve repeated, continuing treatment in order to achieve long-term tumor suppression and feline angiostatin allows for the treatment of cat tumors using specie-

specific angiogenesis inhibitors, thereby minimizing the risk of evoking immune responses under repeated administration. Finally, spontaneous feline tumors are very similar to their human correlates in histopathologic and biologic behavior (MacEwen, 1990, *Cancer Metastasis Rev* 9(2): 125-36), therefore experimental results obtained from feline tumors will also provide valuable information for human cancer biology and treatment.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:
 - 5 a) a nucleotide sequence that encodes an angiotensin, wherein said angiotensin comprises the polypeptide as shown in SEQ ID NO: 2 or SEQ ID NO: 4;
 - b) a nucleotide sequence comprising SEQ ID NO: 1 or SEQ ID NO: 3;
 - c) a nucleotide sequence which hybridizes to the complement of the nucleotide sequence of a) or b), with the proviso that the nucleotide sequence does not
 - 10 encode a chicken, human or mouse angiotensin; and
 - d) the complement of a nucleotide sequence selected from a), b) or c).
2. A vector comprising the nucleic acid of claim 1.
- 15 3. A host cell genetically engineered to contain the nucleic acid of claim 1.
4. The host cell of Claim 3 wherein said nucleic acid is operatively linked with a regulatory nucleotide sequence capable of directing the expression of the polypeptide encoded by said nucleic acid in said host cell.
- 20 5. A transgenic, non-human animal which has been genetically engineered to contain a transgene comprising the nucleic acid of claim 1.
6. An isolated polypeptide comprising the amino acid sequence as set forth in SEQ ID
- 25 NO: 2 or SEQ ID NO: 4.
7. An isolated polypeptide comprising an amino acid sequence encoded by the isolated nucleic acid molecule of claim 1.
- 30 8. An antibody which binds to the isolated polypeptide of claim 6 or 7.
9. An isolated fusion polypeptide comprising a fusion peptide and an amino acid sequence selected from SEQ ID NO: 2, SEQ ID NO: 4, or an amino acid sequence encoded by the isolated nucleic acid molecule of claim 1.
- 35 10. A method for treating an angiogenesis-related disorder in a subject comprising administering to the subject a compound which modulates the function, activity and/or expression of an angiotensin in the subject.
- 40 11. The method of claim 10, wherein the angiogenesis-related disorder is selected from

the group consisting of cancer; angiogenesis-dependent cancer comprising solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, comprising hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, comprising diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophilic joints; angiofibroma; wound granulation; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; diabetic neovascularization; macular degeneration; fractures; vasculogenesis; hematopoiesis; ovulation; menstruation; and placentation.

12. A method for identifying a compound which modulates expression of an angiostatin sequence comprising:

- bringing a test compound into contact with a cell that expresses an angiostatin;
- measuring a level of expression of said angiostatin sequence in the cell;
- comparing the level of angiostatin sequence expression in the cell in the presence of the test compound to a level of angiostatin sequence expression in the cell in the absence of the test compound,

wherein if the level of angiostatin sequence expression in the cell in the presence of the test compound differs from the level of expression of the angiostatin sequence in the cell in the absence of the test compound, a compound that modulates expression of an angiostatin is identified.

13. A method for identifying a compound which modulates activity of an angiostatin comprising:

- contacting a test compound to a cell that expresses an angiostatin;
- measuring the level of activity of the said angiostatin in the cell;
- comparing the level of activity of said angiostatin in the cell in the presence of the test compound to the level of activity of said angiostatin in the cell in the absence of the test compound,

wherein if the level of activity of said angiostatin in the cell in the presence of the test compound differs from the level of angiostatin activity in the cell in the absence of the test compound, a compound that modulates activity of said angiostatin is identified.

14. A method for modulating the activity and/or expression of an angiostatin sequence in a cell comprising administering to the cell a compound which modulates the activity and/or expression of an angiostatin sequence in the cell.

15. The method according to any one of claims 10-14, wherein said angiostatin comprises the amino acid sequence as set forth in SEQ ID NO: 2 or SEQ ID NO: 4.

FIG. 1

1 TGCAGGGCAT TCCAGTACCA CAGTAAAGAG CAACATTGTG TGATCATGGC TGAAAAACAGC
 61 AAGAATTCCG CTGTCTTCAG GATGAGAGAT GTGGTTTAT TTGAAAAAGAA GATCTATCTT
 121 TCAGAGTGCA AGATTGAAA CGGGAAGAGC TACAGGGGA CGATGTCCAA AACGAAGAAT
 181 GGTGTCACCT GTCAAAAATG GAGTGACAAT GCTCCGCACA AACCTAATA TTCACCTGAT
 241 AAGTACCCCTG CGAGGGGCT GTACACCCAC AGACCCAAAC AAGAGTTCTG ACTACTGCCA GATCCAGAG
 301 GGACCCCTGGT GCTACACCCAC AATGTATGCA CTGCAGTGGG GAAAACTATG AGGCAGAAAT TTCCAAGACC
 361 TGTGAAGATG AATGTATGCA CTGCAGTGGG GAAAACTATG AGGCAGAAAT TTCCAAGACC
 421 ATCTCTGGAA TCAAAATGCCA GTCCCTGGAAC TCTCAAACCC CACATGCTCA CGGATATATT
 481 CCTTCCAAAT TTCCAAACAA GAACCTGAAG ATGAATTACT GCCGTAACCC TGATGGGGAG
 541 CCCC GCCCGCCGT GGTGTTTCAC CACAGATCCC AACAAACGCT GGGAGTTCTG TGACATCCCCG
 601 CGCTGTACAA CACCACCCCC AACTCCTGGT CCAACGTATG AGTGTCTGAA AGGCAAGGT
 661 GAAACTACC GAGGGAAGT GTCCCTCACC GTATCTGGAC ACATTGTCA GCGCTGGAGC
 721 GAACAGACCC CTCACAAGCA CAACAGGACC CCTGAAAAC TCCCTTGCAA AAATTGGAT
 781 GAAACTACT GTCGCAACCC TGATGGAGAA ACGACTCCGT GGTGCTATAC GACCAACAGC
 841 GAAGTGAGGT GGAATACTG TCAGATCCCG TCCTGTGAGT CCTCGCCACT GTCTCAGAA
 901 CATTTGGACG CCCAGTTTC AGTACCACCT GAACAAACTC CCGTGGTCCA GGAGTGCTAC
 961 CGAGGCAACG GACAGATTG TCGAGGCACA TCATCTACTA CTATCACAGG AAAGAAGTGT
 1021 CAACCTTGGT CATCTATGGT ACCACACCGA CATGTGAAGA CCCCAGAACG CTACCCAGAC
 1081 GCCGGCCTGA CCATGAAC TA TGTAACCTGA AAAATGTCC GGACAGAGAA
 1141 ACCACCGACC CCAAGTCCG GACTGTTCC CAGGTTCCGA GTGCAGAGGA CCTTCCGAG
 1201 GAGAGTGCCA CCAAGTCCG GACTGTTCC CAGGTTCCGA GTGCAGAGGA CCTTCCGAG
 1261 TCAGACTGCA TGTTCGGGA TGGCAAGGA TACCGGGCA AGAGGCAAC CACCGTTTCG
 1321 GGCATCCCTT GCCAGGAGTG GGGAGCCCAG GAGCCCCACA GACACAGCAT

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FIG. 2

C R A F Q Y H S K E Q H C V I M A E N S
1 TGCAGGGCATTCCAGTACCACAGTAAAGAGCAACATTGTGTGATCATGGCTGAAAACAGC
K N S A V F R M R D V V L F E K K I Y L
61 AAGAATTCGGCTGTCTTCAGGATGAGAGATGTGGTTTATTTGAAAAGAAGATCTATCTT
S E C K I G N G K S Y R G T M S K T K N
121 TCAGAGTGCAAGATTGGAAACGGGAAGAGCTACAGGGGGACGATGTCCAAAACGAAGAAT
G V T C Q K W S D N A P H K P N Y S P D
181 GGTGTCACCTGTCAAAAATGGAGTGACAATGCTCCGCACAAACCTAACTATTCACCTGAT
K Y P A E G L E E N Y C R N P D S D E D
241 AAGTACCCTGCGGAGGGGCTGGAGGAGAATTACTGCAGGAACCCCGACAGTGACGAGGAC
G P W C Y T T D P N K R F D Y C D I P E
301 GGACCTGGTGCTACACCACAGACCCAAACAAGAGGTTGACTACTGCGACATTCCAGAG
C E D E C M H C S G E N Y R G K I S K T
361 TGTGAAGATGAATGTATGCACTGCAGTGGGGAAAACCTATGAGGGCAAAATTTCCAAGACC
I S G I K C Q S W N S Q T P H A H G Y I
421 ATCTCTGGAATCAAATGCCAGTCTGGAACCTCTCAAACCCACATGCTCACGGATATATT
P S K F P N K N L K M N Y C R N P D G E
481 CCTTCCAAATTTCCAAACAAGAACCTGAAGATGAATTACTGCCGTAACCTGATGGGGAG
P R P W C F T T D P N K R W E F C D I P
541 CCCCCTGGTGTTCACACAGATCCCAACAACGCTGGGAGTTCTGTGACATCCCCG
R C T T P P P T P G P T Y E C L K G K G
601 CGCTGTACAACACCACCCCAACTCTGGTCCAACGTATGAGTGTCTGAAAGGCAAGGT
E N Y R G K V S L T V S G H T C Q R W S
661 GAAACTACCGAGGGAAGGTGTCCCTCACCGTATCTGGACACACTGTGACGCTGGAGC
E Q T P H K H N R T P E N F P C K N L D
721 GAACAGACCCCTCACAAGCACAACAGGACCCCTGAAAACCTCCCTTGCAAAAATTTGGAT
E N Y C R N P D G E T T P W C Y T T N S
781 GAAACTACTOTCGCAACCTGATGGAGAAACGACTCCGTGGTGTATACGACCAACAGC
E V R W E Y C Q I P S C E S S P L S S E
841 GAAGTGAGGTGGGAATACTGTGAGATCCCGTCTGTGAGTCTCGCCACTGTCTCAGAA
H L D A P V S V P P E Q T P V V Q E C Y
901 CATTTGGACGCCCCAGTTTCAGTACCACCTGAACAAACTCCCGTGGTCCAGGAOTGCTAC
R G N G Q S Y R G T S S T T I T G K K C
961 CGAGGCAACGGACAGAGTTATCGAGGCACATCATCTACTATCACAGGAKAGAAGTGT
Q P W S S M V P H R H V K T P E R Y P D
1021 CAACCTTGGTCATCTATGGTACCACACCGACATGTGAAGACCCCAAGACGCTACCCAGAC
A G L T M N Y C R N P D A D K S P W C Y
1081 GCCGGCTGACCATGAACTACTGCAGGAACCCAGACGCCGACAAGAGCCCTTGGTGTAC
T T D P S V R W E F C N L K K C P D R E
1141 ACCACCGACCCAGTGTCCGCTGGGAATTCTGTAACCTGAAAAATGTCCGGACAGAGAA
E S A T K S P T V S Q V P S A E D P S E
1201 GAGAGTGCCACCAAGTCCCCGACTGTTTCCAGGTTCCGAGTGCAGAGGACCCCTCCGAG
S D C M F G N G K G Y R G K R A T T V S
1261 TCAGACTGCATGTTTCGGGAATGGCAAAGGATACCGGGGCAAGAGGGCAACCACCGTTTCG
G I P C Q E W G A Q E P H R H S
1321 GGCATCCCCTGCCAGGAGTGGGGAGCCCAGGAGCCCCACAGACACAGCAT

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FIG. 3

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1  ATCTATCTTT CAGAGTGCAA GATTGGAAC GGAAGAGCT ACAGGGGAC GATGTCCAA
61  ACGAAGAATG GTGTCACCTG TCAAAATGG AGTGACAATG CTCCGCACAA ACCTAACTAT
121  TCACCTGATA AGTACCCCTG GAGGGGCTG GAGGAGAAAT ACTGCAGGAA CCCCGACAGT
181  GACGAGGACG GACCCCTGGT GTGAAGATGA ATGTATGCAC TGCACTGGGG AAAACTATGA CTACTGCGAC
241  ATTCCAGAGT TCTCTGGAAT TCTCCAAAT TCCAAACAAG AACCTGAAGA TGAATTAATG CCGTAACCCCT
301  TCCAAGACCA GGATATATTC CCGGCCGTG GTGTTTCACC ACAGATCCCA ACAACGCTG GGAGTTCTGT
361  GATGGGGAGC GCTGTACAA GCTGTACAA ACCACCCCA ACTCCTGGT CAACGTATGA GTGTCTGAAA
421  GACATCCCGC AAAACTACCG AGGGAAGGTG TCCCTCACCG TATCTGGACA CACTTGTCAAG
481  GGCAAGGTG AACAGACCC AACAGACCC TCGCAACCC TATGGAGAAA CGACTCCGTG GTGCTATACG
541  CGCTGGAGCG AACTTGGATG AAGTGAGGTG GGAATACTGT GTACCCACTG AACAAACTCC CGTGGTCCAG
601  AATTGGATG AAGTGAGGTG GGAATACTGT GTACCCACTG AACAAACTCC CGTGGTCCAG
661  ACCAACAGCG AATTGGACGC CCCAGTTTCA GTACCCACTG CGAGGCACAT CATCTACTAC TATCACAGGA
721  TCCTCAGAAC ATTGGACGC CCCAGTTTCA GTACCCACTG CGAGGCACAT CATCTACTAC TATCACAGGA
781  GAGTGTACG GAGGCAACGG ACAGAGTTAT CGAGGCACAT CATCTACTAC TATCACAGGA
841  AAGAAGTGTG AACCTTGGT ATCTATGGTA CCACACCGAC ATGTGAAGAC CCCAGAACGC
901  TACCCAGACG CCGGCTGAC CATGAACTAC TGCAGGAACC CAGACGCCCG CAAGAGCCCT
961  TGGTGTTACA CCACCGACCC CAGTGTCCGC TGGGAATCT GTAACTCTG AAAATGTCCG
1021  GACAGAGAAG AG
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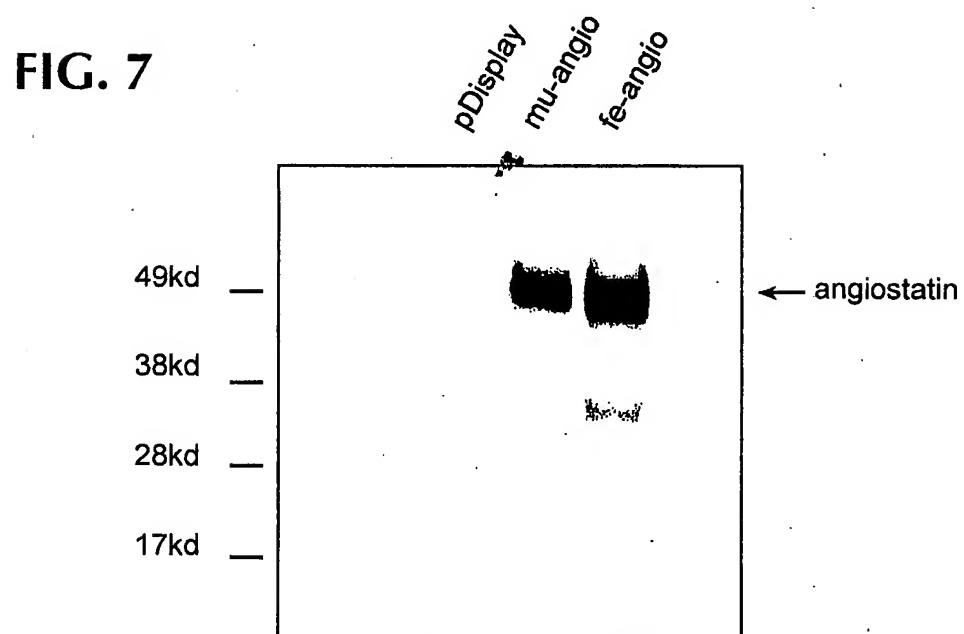
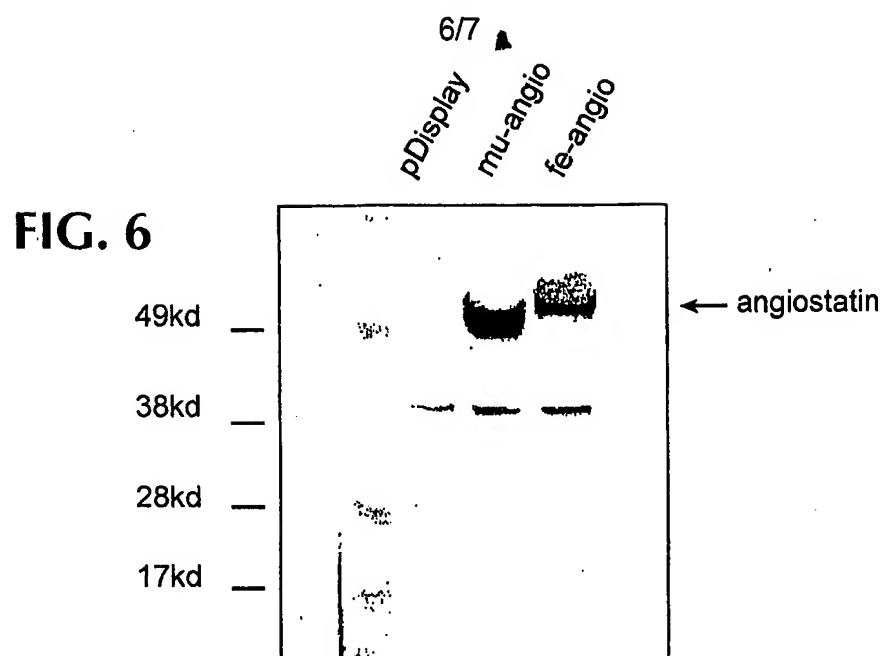
FIG. 4

I Y L S E C K I G N G K S Y R G T M S K
1 ATCTATCTTTCAGAGTGCAAGATTGGAAACGGGAAGAGCTACAGGGGACGATGTCCAAA
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61 ACGAAGAATGGTGTACCTGTCAAAAATGGAGTGACAATGCTCCGCACAAACCTAACTAT
S P D K Y P A E G L E E N Y C R N P D S
121 TCACCTGATAAGTACCCTGCCGAGGGGCTGGAGGAGAATTACTGCAGGAACCCCGACAGT
D E D G P W C Y T T D P N K R F D Y C D
181 GACGAGGACGGACCCTGGTGTACACCACAGACCCAAACAAGAGGTTTCGACTACTGCGAC
I P E C E D E C M H C S G E N Y E G K I
241 ATTCCAGAGTGTGAAGATGAATGTATGCTGCTGAGTGGGGAAAACCTATGAGGGCAAAATT
S K T I S G I K C Q S W N S Q T P H A H
301 TCCAAGACCATCTCTGGAATCAAATGCCAGTCCTGGAACCTCAAACCCACATGCTCAC
G Y I P S K F P N K N L K M N Y C R N P
361 GGATATATTCTTCCAAATTTCCAAACAAGAACCTGAAGATGAATTACTGCCGTAACCCCT
D G E P R P W C F T T D P N K R W E F C
421 GATGGGGAGCCCCGCGTGGTGTTCACCACAGATCCCAACAAACGCTGGGAGTTCTGT
D I P R C T T P P P T P G P T Y E C L K
481 GACATCCCGCTGTACAACACCACCCCAACTCCTGGTCCAACGTATGAGTGTCTGAAA
G K G E N Y R G K V S L T V S G H T C Q
541 GGCAAAGGTGAAAACCTACCGAGGGAAGGTGTCCCTCACCGTATCTGGACACACTTGTGAG
R W S E Q T P H K H N R T P E N F P C K
601 CGCTGGAGCGAACAGACCCCTCACAAGCACAACAGGACCCCTGAAAACCTTCCCTTGCAAA
N L D E N Y C R N P D G E T T P W C Y T
661 AATTTGGATGAAAACCTACTGTGCAACCCTGATGGAGAAACGACTCCGTGGTGTCTATACG
T N S E V R W E Y C Q I P S C E S S P L
721 ACCAACAGCGAAGTGAGGTGGGAATACTGTGTCAGATCCCGTCTGTGAGTCTCGCCACTG
S S E H L D A P V S V P P E Q T P V V Q
781 TCCTCAGAACATTTGGACGCCCCAGTTTCAGTACCACCTGAACAAACCTCCCGTGGTCCAG
E C Y R G N G Q S Y R G T S S T T I T G
841 GAGTGCTACCGAGGCAACGGACAGAGTTATCGAGGCACATCATCTACTACTATCACAGGA
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901 AAGAAGTGTCAACCTTGGTTCATCTATGGTACCACACCGACATGTGAAGACCCGAGAACGC
Y P D A G L T M N Y C R N P D A D K S P
961 TACCCAGACGCGGCTGACCATGAACTACTGCAGGAACCCAGACGCGGACAAGAGCCCT
W C Y T T D P S V R W E F C N L K K C P
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D R E E
1081 GACAGAGAAGAG

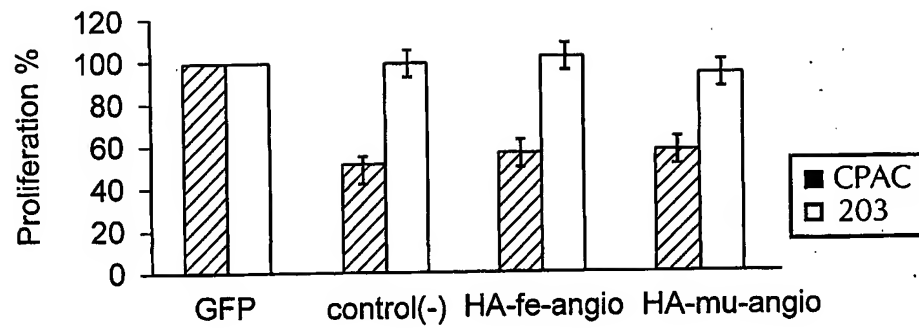
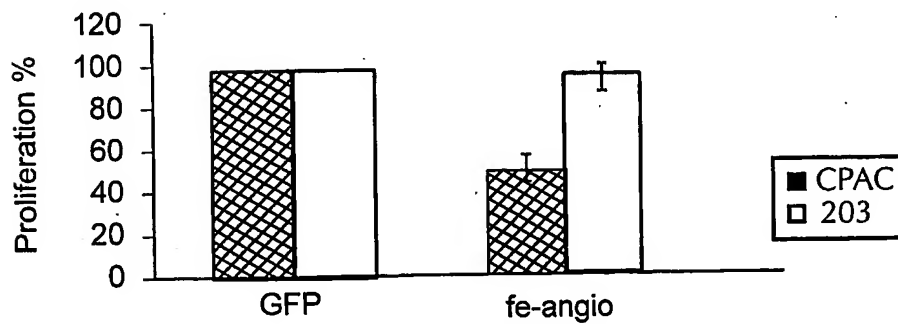
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FIG. 5

1	LYLSECKTIGNGKSYRGTMSTKNGVTCQKWSNAPHKPNY	angiostatin-feline
1	VYLSECKTGNGKINRGTMSTKNGITCQKWSSTSPHRRF	angiostatin-human
1	VYLSECKTGIGNGYRGTMSTRTKSGVACQKWGATFPHVPNY	angiostatin-mouse
1	LYLLECKTGNGQTYRGITAEKTSQVTCQKWSATSPHVFKE	angiostatin-bovine
41	SPDKYPAEGLEENYCRNPDSDGSPWCYTTPDKRFDYCD	angiostatin-feline
41	SPATHPEGLEENYCRNPDNDPQGPWCYTTPDEKRYDYCD	angiostatin-human
41	SPSTHPNEGLEENYCRNPDNDEQGPWCYTTPDPKRYDYCN	angiostatin-mouse
41	SPEKFFLAGLEENYCRNPDNDENGFWCYTTPDPKRYDYCD	angiostatin-bovine
81	IPECEDECMHCSGENYEGKISKITISGLKCSWNSQTPHAH	angiostatin-feline
81	ILECEDECMHCSGENYDGKISKTMGLCQAWDSQSPHAH	angiostatin-human
81	IPECEDECMYCSGEEKYEGKISKTMGLDCQAWDSQSPHAH	angiostatin-mouse
81	IPECEDKCMHCSGENYEGKIAKTMSEKDCQAWDSQSPHAH	angiostatin-bovine
121	GYIPSKFPKNLKMNYCRNPDGEPRPWCFTTDPNKRWEFC	angiostatin-feline
121	GYIPSKFPKNLKENYCRNPDFTIRPWCFTTDPNKRWEIC	angiostatin-human
121	GYIHAKFHSKNLKMNYCHNPDGEPRPWCFTTDPNKRWEYC	angiostatin-mouse
121	GYIPSKFPKNLKMNYCRNPDGEPRPWCFTTDPNKRWEFC	angiostatin-bovine
161	DIPRCTTPPPTEGPTYECLKGGKGENYRGKMSLTVSGHTCQ	angiostatin-feline
161	DIPRCTTPPPSSGPTYQCLKGTGENYRGNAVTVSGHTCQ	angiostatin-human
161	DIPRCTTPPPPPSPTYQCLKGRGENYRGTVSVTVSGKTCQ	angiostatin-mouse
161	DIPRCTTPPPSSGHKYQCLKGTGKNYGGTVAVTESGHTCQ	angiostatin-bovine
201	RWSEQTPHKNRTPENFPCKNLDENYCRNPDGETTPWCYT	angiostatin-feline
201	RWSAQTPHTNRTPENFPCKNLDENYCRNPDGKRAPWCHT	angiostatin-human
201	RWSEQTPHKNRTPENFPCKNLDENYCRNPDGETAPWCYT	angiostatin-mouse
201	RWSEQTPHKNRTPENFPCKNLDENYCRNPDGKRAPWCYT	angiostatin-bovine
241	TNSEVRWEYCQIPSCSSPLSSEHLDAPVSVPEQTPVVQ	angiostatin-feline
241	TNSQVRWEYCKIPSDSSHVSTEQAPT--APPELLTPVVQ	angiostatin-human
241	TDSQVRWEYCEIPSCSSASPDQSDSSV--PPEEQTPVVQ	angiostatin-mouse
241	TNSEVRWEYCTIPSCSSPLSTERNMVE--VPPEQTPVPEQ	angiostatin-bovine
281	ECYRNGQSYRGTSSTTITGKKCQFWSSMVPHRHVKTPEH	angiostatin-feline
279	DCYHGDGQSYRGTSSTTITGKKCQSWSSMTPHRHOKTPEN	angiostatin-human
279	ECYQSDGQSYRGTSSTTITGKKCQSWAAMFPHRHOKTPEN	angiostatin-mouse
279	DCYHGNQSYRGTSSTTITGRKCQSWSSMTPHRHLKTPEN	angiostatin-bovine
321	YPDAGLTMNYCRNPDADKSPWCYTTPSVRWEFCNLKKCP	angiostatin-feline
319	YHNAGLTMNYCRNPDADKGPWCFTTTPSVRWEFCNLKKCS	angiostatin-human
319	HPDAGLEMNCRNPDGDKGPWCYTTPSVRWEFCNLKKCS	angiostatin-mouse
319	YHNAGLTMNYCRNPDADKSPWCYTTPHVRWEFCNLKKCS	angiostatin-bovine
361	DREE	angiostatin-feline
359	GTEA	angiostatin-human
359	ETEG	angiostatin-mouse
359	ETPE	angiostatin-bovine



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FIG. 8**FIG. 9**

SEQUENCE LISTING

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 1 5 10 15

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 20 25 30

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Lys Ser Tyr Arg Gly Thr Met Ser Lys Thr Lys Asn Gly Val Thr Cys
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Gln Lys Trp Ser Asp Asn Ala Pro His Lys Pro Asn Tyr Ser Pro Asp
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Lys Tyr Pro Ala Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp
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Ser Asp Glu Asp Gly Pro Trp Cys Tyr Thr Thr Asp Pro Asn Lys Arg
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Phe Asp Tyr Cys Asp Ile Pro Glu Cys Glu Asp Glu Cys Met His Cys
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Ser Gly Glu Asn Tyr Glu Gly Lys Ile Ser Lys Thr Ile Ser Gly Ile
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Lys Cys Gln Ser Trp Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile
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Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn
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Pro Asp Gly Glu Pro Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys
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Arg Trp Glu Phe Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Thr
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Pro Gly Pro Thr Tyr Glu Cys Leu Lys Gly Lys Gly Glu Asn Tyr Arg
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 Gly Lys Val Ser Leu Thr Val Ser Gly His Thr Cys Gln Arg Trp Ser
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 Glu Gln Thr Pro His Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys
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 Lys Asn Leu Asp Glu Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Thr
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 Pro Val Ser Val Pro Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr
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 Arg Gly Asn Gly Gln Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr
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 Gly Lys Lys Cys Gln Pro Trp Ser Ser Met Val Pro His Arg His Val
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 Lys Thr Pro Glu Arg Tyr Pro Asp Ala Gly Leu Thr Met Asn Tyr Cys
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 Arg Asn Pro Asp Ala Asp Lys Ser Pro Trp Cys Tyr Thr Thr Asp Pro
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 Ser Val Arg Trp Glu Phe Cys Asn Leu Lys Lys Cys Pro Asp Arg Glu
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 Glu Ser Ala Thr Lys Ser Pro Thr Val Ser Gln Val Pro Ser Ala Glu
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 Asp Pro Ser Glu Ser Asp Cys Met Phe Gly Asn Gly Lys Gly Tyr Arg
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Asn Ala Pro His Lys Pro Asn Tyr Ser Pro Asp Lys Tyr Pro Ala Glu
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Gly Leu Glu Glu Asn Tyr Cys Arg Asn Pro Asp Ser Asp Glu Asp Gly
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Pro Trp Cys Tyr Thr Thr Asp Pro Asn Lys Arg Phe Asp Tyr Cys Asp
 65 70 75 80

Ile Pro Glu Cys Glu Asp Glu Cys Met His Cys Ser Gly Glu Asn Tyr
 85 90 95

Glu Gly Lys Ile Ser Lys Thr Ile Ser Gly Ile Lys Cys Gln Ser Trp
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Asn Ser Gln Thr Pro His Ala His Gly Tyr Ile Pro Ser Lys Phe Pro
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Asn Lys Asn Leu Lys Met Asn Tyr Cys Arg Asn Pro Asp Gly Glu Pro
 130 135 140

Arg Pro Trp Cys Phe Thr Thr Asp Pro Asn Lys Arg Trp Glu Phe Cys
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Asp Ile Pro Arg Cys Thr Thr Pro Pro Pro Thr Pro Gly Pro Thr Tyr
 165 170 175

Glu Cys Leu Lys Gly Lys Gly Glu Asn Tyr Arg Gly Lys Val Ser Leu
 180 185 190

Thr Val Ser Gly His Thr Cys Gln Arg Trp Ser Glu Gln Thr Pro His
 195 200 205

Lys His Asn Arg Thr Pro Glu Asn Phe Pro Cys Lys Asn Leu Asp Glu
 210 215 220

Asn Tyr Cys Arg Asn Pro Asp Gly Glu Thr Thr Pro Trp Cys Tyr Thr
 225 230 235 240

Thr Asn Ser Glu Val Arg Trp Glu Tyr Cys Gln Ile Pro Ser Cys Glu
 245 250 255

Ser Ser Pro Leu Ser Ser Glu His Leu Asp Ala Pro Val Ser Val Pro
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Pro Glu Gln Thr Pro Val Val Gln Glu Cys Tyr Arg Gly Asn Gly Gln
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Ser Tyr Arg Gly Thr Ser Ser Thr Thr Ile Thr Gly Lys Lys Cys Gln
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Pro Trp Ser Ser Met Val Pro His Arg His Val Lys Thr Pro Glu Arg
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Tyr Pro Asp Ala Gly Leu Thr Met Asn Tyr Cys Arg Asn Pro Asp Ala
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38

INTERNATIONAL SEARCH REPORT

International Application No
PCT/162004/001802

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, BIOSIS, EMBASE, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 197 550 A (PFIZER PROD INC) 17 April 2002 (2002-04-17) the whole document	1-15
X	WO 98/54217 A (CHILDRENS MEDICAL CENTER) 3 December 1998 (1998-12-03) see whole doc. esp. claims and examples	1-15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the International search

25 October 2004

Date of mailing of the International search report

04/11/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mueller, F

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2004/001802

Box II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 10,11 and 14,15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte
l Application No
PCT/IB2004/001802

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EP 1197550	A	17-04-2002	EP 1197550 A2	17-04-2002
			JP 2002355056 A	10-12-2002
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WO 9854217	A	03-12-1998	US 5945403 A	31-08-1999
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			JP 2001506506 T	22-05-2001
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			US 2001029246 A1	11-10-2001